Distribution and quantitative estimates of variant Creutzfeldt Jakob Diseases prions in the tissues of clinical and asymptomatic patients

EMERGING INFECTIOUS DISEASES®

Distribution and quantitative estimates of variant Creutzfeldt Jakob Diseases prions in the tissues of clinical and asymptomatic patients

Journal:	*Emerging Infectious Diseases*
Manuscript ID	EID-16-1734.R1
Manuscript Type:	Research
Date Submitted by the Author:	16-Jan-2017
Complete List of Authors:	douet, Jean-Yves; INRA, UMR INRA ENVT 1225 LACROUX, Caroline; UMR INRA ENVT, 1225 Aron, Naima; INRA, UMR INRA ENVT 1225 Lugan, Severine; INRA, UMR INRA ENVT 1225 Head, Mark; University of Edinburgh, National CJD Research & Surveillance Unit tillier, cécile; UMR INRA ENVT, 1225 huor, alvina; UMR INRA ENVT, 1225 cassard, herve; INRA, UMR INRA ENVT 1225 Arnold, Mark; Animal Health and Veterinary Laboratories Agency (AHVLA), Department of Epidemilogical Sciences Beringue, Vincent; INRA, Unité Virologie Immunologie Moléculaires; Ironside, James; Western General Hospital, National CJD Surveillance Unit ANDREOLETTI, Olivier; ENVT, UMR 1225;
Keywords:	Prion, vCJD, public health, infectivity

Distribution and quantitative estimates of variant Creutzfeldt-Jakob disease prions in the tissues of clinical and asymptomatic patients

Jean Yves Douet[1], Caroline Lacroux[1], Naima Aron[1], Mark W Head[2], Séverine Lugan[1], Cécile Tillier[1], Alvina Huor[1], Hervé Cassard[1], Mark Arnold[3], Vincent Beringue[4], James W Ironside[2] and Olivier Andréoletti[1 *]

[1] *UMR INRA ENVT 1225, Interactions Hôtes Agents Pathogènes, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles 31076 Toulouse, France*

[2] *National Creutzfeldt-Jakob Disease Research & Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK*

[3] *APHA Sutton Bonington, Loughborough, Leicestershire, LE12 5NB, UK*

[4] *VIM, INRA, Université Paris-Saclay, 78350, Jouy-en-Josas, France*

Author summary line: A rapid in vitro assay shows that the distribution of vCJD prions in the tissues of clinical and asymptomatic patients is wider than previously thought

Running title: Variant CJD iatrogenic transmission risk

Keywords: Prions, infectivity, public health, vCJD

* Corresponding author: o. andreoletti o.andreoletti@envt.fr

Abstract

In the United-Kingdom about 1 in 2000 people could be infected with variant Creutzfeldt-Jakob Disease (vCJD). The risk of transmission of vCJD by medical procedures therefore remains a major concern for public health authorities.

In this study, we used *in vitro* amplification of prions by Protein Misfolding Cyclic Amplification (PMCA) to estimate the distribution and the level of the vCJD agent in a range of tissues (n=21) from four patients who died with clinical vCJD and from one asymptomatic vCJD-infected individual.

Unexpectedly, PMCA demonstrated the presence of significant levels of vCJD prions in a wide range of tissues, including liver, salivary gland, kidney, lung or bone marrow. Bioassays confirmed that the quantitative estimate of vCJD prion accumulation levels provided by PMCA are indicative of vCJD infectivity levels in tissues.

This work provides critical data for the design of rational measures aimed at minimising the risk of iatrogenic transmission of vCJD.

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that occur naturally in sheep (scrapie), cattle (bovine spongiform encephalopathy - BSE), and humans (Creutzfeldt-Jakob disease - CJD). A key event in the pathogenesis of TSEs is the conversion of the normal cellular prion protein (PrP^{C}, encoded by *PRNP*) into an abnormal disease-associated isoform (PrP^{Sc}) in tissues of infected individuals. PrP^{C} is completely degraded after controlled digestion with proteinase K (PK) in the presence of non-denaturing detergents. In contrast, PrP^{Sc} is N-terminally truncated under the same conditions, leaving a PK resistant core, termed PrP^{res} (1).

In 1996, a new form of CJD, termed variant CJD (vCJD) was identified in the UK, which is believed to result from zoonotic transmission of the BSE agent, probably as a consequence of dietary exposure to BSE-contaminated meat products (2, 3). The total number of clinical cases of vCJD identified thus far is limited (227 patients worldwide at the time of writing). However, the estimated prevalence of asymptomatic vCJD infection in populations exposed to the BSE agent is uncertain (4). In the UK 32,441 appendix samples, collected during surgery on patients born between 1941 and 1985 have been tested for abnormal prion protein accumulation by immunohistochemistry. This study gave a vCJD prevalence estimate of 1 in 2,000 in these age cohorts (95% Confidence Interval ranging from 1 in 3,500 to 1 in 1,250) (5). There is no comparable data available concerning the prevalence of asymptomatic vCJD infection in other countries, although BSE exposure is known to have occurred in several continental European counties, as judged by cases of vCJD that are not attributable to exposure in the UK (http://www.cjd.ed.ac.uk/documents/worldfigs.pdf).

Over the last two decades several studies have reported on the distribution of the vCJD agent in the tissues of infected patients (6-8). Most have failed to detect the vCJD agent outside the nervous system (central, peripheral and autonomic) and lymphoid tissues. However, the

sensitivity of the PrP^{res} detection techniques used in these historical investigations was limited.

The Protein Misfolding Cyclic Amplification (PMCA) is thought to mimic prion replication *in vitro*, but in an accelerated form, allowing amplification of minute amounts of PrP^{Sc} and prion infectivity (9). In PMCA, a PrP^{C}-containing "substrate" is combined with a "seed" that may contain otherwise undetectable amounts of PrP^{Sc}. Following repeated cycles of incubation and sonication, the amount of PrP^{Sc} increases to levels at which they can be detected by conventional biochemical means. Recently, we and others have shown that PMCA is able to detect endogenous vCJD agent present in patient biological fluids such as urine and blood (10, 11).

In this study, we first evaluated the relative sensitivity of the PMCA assay versus bioassay in mice for the detection of the vCJD agent. We then estimated, using PMCA, the level of vCJD prions in 21 tissues collected from patients who died with symptomatic vCJD (n=4) and from a patient with an asymptomatic vCJD infection. Finally, we determined whether the vCJD prion levels as estimated by PMCA were consistent with the infectious titres as estimated by bioassay using transgenic mice.

Methods

Ethics Statement

All animal experiments were performed in compliance with institutional and French national guidelines and in accordance with the European Community Council Directive 86/609/EEC. The animal experiments that are part of this study (national registration 01734.01) were approved by the local ENVT ethic committee. Mouse inoculations were performed under anaesthesia (isofulorane). Mice that displayed clinical signs were anesthetized with isofluorane before sacrifice using CO_2 inhalation.

Human samples were obtained from the UK National CJD Research & Surveillance Unit Brain and Tissue Bank, which is part of the MRC Edinburgh Brain Bank. Tissue samples were pseudo-anonymized using a Brain Bank reference number. All UK cases had informed consent for research and their supply and use in this study was covered by the East of Scotland Research Ethics Service approval for the Edinburgh Brain Bank (16/ES/0084).

vCJD and control patients

Tissues from four clinical vCJD cases (referred to in this study as vCJD-1 to vCJD-4) and one asymptomatic vCJD- infected individual who received a transfusion of packed red blood cells from a donor who subsequently died from vCJD (12) (vCJD-A) were investigated. Tissues from two non-vCJD infected patients (NC-1 and NC-2) were used as controls. Basic demographic details for the vCJD and control patients are presented in table 3. In cases with appropriate consent, the entire *PRNP* coding sequence was established as to exclude pathogenic mutations in the *PRNP* gene (13, 14). Table 4 shows the tissue tested from each patient.

Mouse bioassays

Bioassay was carried out using mice expressing bovine PrP (tgBov-Tg100) as previously described (15, 16). These mice were observed daily and their neurological status was assessed weekly. When clinically progressive TSE symptoms were evident, or at the end of their lifespan, the animals were euthanized. Survival time was expressed as the mean of the days post inoculation (dpi) of all the mice scored positive for PrP^{res}, with a corresponding standard deviation. In cages where no clinical signs were observed, mice were sacrificed at the end of their natural life-span (600 to 800 days). In those cases, incubation periods reported in the table as >600 dpi, corresponded to the survival time observed in at least three out of the six mice.

Infectious prion titre estimates

The infectious titre in a reference 10% weight/vol frontal cortex homogenate from a clinical vCJD patient was established by endpoint titration (intracerebral route) in tgBov mice. The infectious titre (LD_{50}/g IC in tgBov) was estimated by the Spearman method.

The titre of prion infectivity in vCJD affected patients bone marrow samples was estimated using the method developed by Arnold *et al.* (17). This model uses both the probability of survival (attack rate at each dilution) and the individual mouse incubation periods at each dilution to estimate infectious load and is thus able to provide more accurate estimation of titre than using either attack rate or incubation period data alone.

PMCA reactions

A transgenic mouse line that expresses ovine $A_{136}R_{154}Q_{171}$ PrP variant PrP^{C} (tgShXI) was used to prepare the PMCA substrate as previously described (18, 19). PMCA amplification was performed as previously described (11). Each PMCA run included a reference vCJD sample (10% brain homogenate) as a control for the amplification efficiency. Unseeded

controls (1 unseeded control for 8 seeded reactions) were also included in each run. For each tested dilution of each sample, at least four replicates were tested in two independent runs. For each sample, the last dilution showing at least 50% of positive replicates (presence of detectable PrP^{res} in the reaction as assessed by WB) was determined.

Abnormal PrP Western blot (WB) detection

PK resistant abnormal PrP extraction (PrP^{res}) and Western blot were performed as previously described (11). Immunodetection was performed using two different monoclonal PrP-specific antibodies, Sha31 (1 µg/ml) (20), and 12B2 (4 µg/ml) (21), which recognize the amino acid sequences YEDRYYRE (145-152), and WGQGG (89-93) respectively.

Paraffin embedded tissue blot

Paraffin embedded tissue was performed as previously described (22, 23).

Results

Sensitivity of vCJD agent detection by PMCA and bioassay

In order to determine the relative sensitivity of PMCA, a reference sample (10% cerebral cortex homogenate from a vCJD affected patient) that had previously undergone end point titration (IC inoculation route –sup table 1) in bovine PrP expressing mice (tgBov) was re-titrated by PMCA.

The amplification of a ten-fold serial dilution of this sample (6 individual replicates per dilution point) demonstrated that four PMCA rounds (24 hours per round *i.e.* 96 hours) were sufficient to reach the maximal sensitivity level of the assay. Additional PMCA rounds improved neither the analytical sensitivity of the assay nor the number of positive replicates (table 2 –figure 1). Based on these results, the seeding activity (SA) of the isolate was estimated by the Spearman method to be 10^{11} SA_{50} per gram. The bioassay end point titration data of the same sample in tgBov gave an infectious titre of $10^{7.7}$ LD_{50} per gram. Taking into account the 4 fold lower amount of material used to seed the PMCA reaction compared to the material used in mouse inoculations, these results indicate that the PMCA protocol we used was 465 times more sensitive than bioassay in tgBov mice for the detection of vCJD prions.

PMCA results in control and vCJD affected patients

A ten-fold dilution series of the 10% homogenates from the vCJD affected and non-vCJD control patients was then prepared (table 3) and these were subjected to four rounds of PMCA. The amplification products from each round were tested for the presence of PrP^{res} by Western Blot (table 4 and figure 2).

None of the reactions seeded with tissue homogenates from the non-CJD controls were found to be positive for PrP^{res} (table 4). In contrast, the PMCA reactions seeded with tissues from the four symptomatic vCJD patients were found to be positive for PrP^{res} (table 4- figure 2). As

expected, among the tested tissues, the brain homogenates (temporal cortex) showed the highest seeding activity (last PrP^{res} positive dilution 10^{-8}). All of the tested lymphoid organs also displayed seeding activity, but the last PMCA positive dilution varied according to the tested organs from 10^{-2} (thymus) to 10^{-6} (distal ileum and tonsil). Moreover, for a given lymphoid organ, up to a 10^2 fold differences was observed in seeding activity, depending on the patient and sample tested. Taken together these data indicate that in symptomatic vCJD patients the lymphoid organs contain between 10^2 and 10^6 fold less prion seeding activity than the same amount of brain tissue (table 4).

Salivary gland, adrenal gland, liver and bone marrow from the four symptomatic vCJD patients gave positive reactions in PMCA (figure 2 and 3). Using the last dilution to show a positive reaction as a measure of the seeding activity, the vCJD agent in these tissue was found to be 10^3 to 10^6 fold lower than that found in the brain. PrP^{res} was also detected in PMCA reactions seeded with heart, liver, kidney, skeletal muscle, several endocrine/exocrine glands (pancreas, thyroid) and the gonads, from some, but not all, of the four clinical vCJD patients. Where positive, these tissue contained a level of vCJD seeding activity that was equivalent to those observed in distal ileum (*i.e* 10^3 to 10^6 fold lower than in the brain). Irrespective of the tissue used to seed the PMCA reactions, the PrP^{res} Western blot profile in positive reactions was indistinguishable from that observed in reactions seeded with the vCJD brain control (figure 3).

Analysis of tissues from an asymptomatic vCJD-infected individual

No prion seeding activity was detected in the brain (temporal cortex) of this asymptomatic vCJD infected patient, who was a *PRNP* codon 129 heterozygote (Met/Val_{129}) (12) (table 4, figure 2). PMCA reactions seeded with dorsal root ganglia or trigeminal ganglia homogenates

from this patient also tested negative. However, seeding activity was detected in the pituitary gland (last PrP^{res} positive dilution 10^{-2}). Additionally, as with the symptomatic vCJD patients, PMCA amplification readily detected the presence of vCJD prions in all of the tested lymphoid organs in this asymptomatic individual. According to the PMCA results, the vCJD agent load in lymphoid organs in this *PRNP* codon 129 Met/Val_{129} asymptomatic patient were similar to those observed in the Met/Met_{129} patients at the clinical stage of the disease. In addition to the lymphoid organs, prion seeding activity was detectable in certain peripheral tissues from this patient (notably salivary gland, lung and liver) (table 4, figure 2 and 3). Strikingly, certain tissues that contained a substantial prion seeding activity in the clinically affected patients (such as bone marrow or the adrenal gland) were found to be negative. Again, the PrP^{res} Western blot profile in positive reactions was indistinguishable from that observed in reactions seeded with the vCJD brain control

vCJD infectivity in bone marrow

In order to test whether the PMCA seeding activity in peripheral tissues of vCJD patients correlated to the presence of infectivity, the bone marrow samples from the symptomatic patient were inoculated to tgBov mice. A clinical TSE was observed in mice that were inoculated with each of the four bone marrow samples. The PrP^{res} Western blot profile and the PrP^{res} distribution pattern as assessed by PET Blot in the brain of the bone marrow-inoculated mice were identical to those observed in tgBov mice inoculated with the vCJD brain control (figure 4).

The data collected in mice inoculated with the bone marrow samples were also used to estimate prion infectivity levels in these samples. For this purpose, we applied the method developed by Arnold *et al.* (17). The approach combines the probability of survival (attack rate) and the individual mouse incubation period to provide an estimation of the infectious

222 titres. Data corresponding to endpoint titration in tgBov mice of the reference vCJD sample
223 (frontal cortex from a clinical vCJD patient) (table 1) were used to derive the relationship
224 between the prion titre of inoculum and the probability of infection and the length of the
225 incubation period (figure 5). According to this model the bone marrow samples displayed an
226 infectious titre that was estimated to range between $10^{2.3}$ and $10^{4.7}$ LD_{50}/g in tgBov (table 5).
227 These values are consistent with a 10^3 to 10^5 lower infectivity load in bone marrow samples
228 than in the reference vCJD brain sample. Interestingly, and in keeping with the PMCA results
229 (table 4), the prion load in bone marrow samples (last PrP^{res} positive dilution 10^{-3} to 10^{-5}) were
230 also 10^3 to 10^5 fold lower than in this reference vCJD isolate (last PrP^{res} positive dilution 10^{-8}).
231 Taken together these results strongly support the contention that the PMCA seeding activity
232 provides a reliable estimate of the prion load in the tissues of vCJD-infected patients.
233
234

235 **Discussion**

236 While most of the previous investigations carried out on tissue from vCJD patients have failed
237 to identify consistent accumulation of the vCJD agent outside the nervous and the lympho-
238 reticular systems, the data obtained in this study clearly demonstrate the presence of vCJD
239 prions in a wide and unexpected variety of peripheral tissues.

240 Natural scrapie and experimental BSE in sheep are two models of orally transmitted prion
241 diseases (24, 25). In both diseases, the agent accumulates in the lympho-reticular system and
242 in the enteric nervous system during the early preclinical phase of the incubation period.
243 Moreover, an early and persistent prionemia is observed in asymptomatic infected animals
244 (26, 27). These features were also observed in vCJD in humans and in view of the likely
245 origin of vCJD (oral exposure to BSE agent), these similarities have led to a consensus that
246 BSE and scrapie in sheep and vCJD in human share a common pathogenesis (28).

247 While the presence of vCJD prions in a variety tissues such as bone marrow, kidney, salivary
248 gland, the skeletal muscle, the pancreas, the liver or the heart might appear surprising, each of
249 these tissue has already been demonstrated to accumulate prion infectivity and/or abnormal
250 prion protein in TSE infected sheep (29-33). Since low levels of infectivity have been
251 reported in blood fractions from a vCJD affected patient, it might be argued such widespread
252 tissue positivity derives from residual blood, rather than from the solid tissue in these samples
253 (16). However, this seems very unlikely given the fact that in whole blood the presence of
254 PMCA amplification inhibitors preclude the detection of endogenous vCJD agent by this
255 methodology (11, 34-36).

256 The *PRNP* codon 129 Met/Val case included in this study is one of only two identified vCJD-
257 infected individuals known to have died of other causes before the onset clinical symptoms of
258 vCJD, and the only case with consent to sample autopsy tissues for research. In this patient,
259 all previous investigations failed to show the presence of abnormal prion protein or infectivity

in the brain (12, 37). The negative PMCA results we obtained using the cerebral cortex, the dorsal root ganglia and the trigeminal ganglia tissue from this patient are consistent with a lack of CNS involvement at the time of death. However, the presence of PMCA seeding activity in the pituitary gland is surprising in this regard.

The presence of abnormal prion protein accumulation in the pituitary gland and other circumventricular organs prior to deposition of PrP^{res} in surrounding brain is a phenomenon that has been reported in TSE infected sheep (38). However, this phenomenon in animals does not seem to represent the main route for neuro-invasion and it is a likely consequence of haematogenous dissemination of the TSE agent through the fenestrated capillary system of the circumventricular organs, which is substantially more permeable than the other capillaries in the brain (the "blood-brain barrier"). This finding may therefore be a consequence of the haematogenous route of secondary vCJD infection in this individual (via transfusion of packed red blood cells from a vCJD-infected donor), in contrast to the oral route of infection in the primary clinical vCJD cases (12).

vCJD prions were detected in certain peripheral tissues from this *PRNP* codon 129 Met/Val patient. Although the distribution of the vCJD seeding activity in the lymphoreticular tissues was similar to that observed in the symptomatic vCJD patients, a number of tissues that were positive in clinically affected patients were negative in this heterozygous asymptomatic individual. These findings suggest that the involvement of some peripheral tissues might occur at a later stage in the incubation period than others, or that they could involve recirculation of the agent from the CNS (*i.e* centrifugal spread at a late state). However, we cannot discount the possibility that that these differences in tissue distribution are due to either the haematogenous route of infection in this individual (as opposed to the likely oral route in the clinical vCJD cases) or the difference between the *PRNP* codon 129 genotype of

the asymptomatic vCJD infected individual (*PRNP* codon 129 Met/Val) and the clinical vCJD cases (*PRNP* codon 129 Met/Met)

Irrespective of the actual explanation for these differences, the presence of vCJD agent in peripheral tissues of patients at both preclinical and clinical stage of the disease indicates the potential for iatrogenic transmission of this fatal neurological condition by surgical procedures. Furthermore, it shows that for certain peripheral tissues a level of infectivity equivalent to an end stage titre (and attendant risk) is reached at a pre-clinical stage.

Several hundred cases of iatrogenic CJD have been reported worldwide. These cases appear to result from the transmission of sporadic CJD (sCJD) and most cases have occurred in the recipients of human dura mater grafts or the administration of human growth hormone extracted from cadaveric pituitaries (39). While in sCJD the distribution of the agent is largely restricted to the nervous system (central and peripheral), the wide distribution of the vCJD agent in the asymptomatic infected patient we describe here may serve to increase the range of medical procedures that might result in the iatrogenic transmission of vCJD (including dentistry, organ transplant, surgery involving non-disposable equipment) (40-43).

Nevertheless, more than 20 years after the identification of the first vCJD patients, only five cases that are a likely consequence of iatrogenic vCJD transmission have come to light, all in the UK and all associated with blood and blood products. These cases were due to either the transfusion of non-leukodepleted red cell concentrates, or with treatment involving large amounts of pooled UK plasma that were known to include donations from individuals who later developed vCJD (12, 44-46). None of the 220 other vCJD cases identified worldwide have been linked to any other medical or dental procedure. Whilst reassuring, it would be unwise to disregard the threat that vCJD still poses for public health. Despite the relatively low number of vCJD clinical cases observed in the United Kingdom (n=178), the most recent

epidemiological studies indicate that 1 out 2000 people in the UK could carry the vCJD agent (as judged by the presence of abnormal prion protein detected by immunohistochemistry in lymphoid follicles in the appendix). Each asymptomatic vCJD-infected person represents a potential source of secondary infection. The data we reported here offers an opportunity for refining the measures that were implemented in many countries to limit the risk of vCJD iatrogenic transmission. The apparent concordance between the PMCA biochemical and infectivity bioassay data, and the higher analytical sensitivity of PMCA suggest that future research need not rely exclusively on time-consuming and costly animal bioassay.

Our results clearly highlight the need for vCJD screening assays. After more than a decade of effort several vCJD blood detection tests have reached a stage in their development that could permit their evaluation as screening or confirmatory assays (11, 47, 48). In particular, there is now a very strong case for the use of PMCA in a highly sensitive and specific blood test for vCJD as judged by our previous work (11, 16) and the very recent publications by Concha-Marambio et al and Bougard et al (35, 36) . The relationship shown here between PrP^{res} amplification by PMCA and the detection of infectivity by bioassay argues that PMCA seeding activity is a good surrogate marker of infectivity, and could provide a sound basis for a vCJD blood test for use on blood or tissue donors.

Acknowledgments

The National CJD Research & Surveillance Unit is supported by the Policy Research Program of the Department of Health and the Scottish Government (DH121/5061). This report is independent research in part funded by the Department of Health Policy Research Programme and the Scottish Government. The views expressed in this publication are those of the author(s) and not necessarily those of the Department of Health or the Scottish Government. The Edinburgh Brain Bank is supported by the Medical Research Council (MRC G0900580). The UMR INRA ENVT 1225 was funded for this work by EU FEDER/INTERREG (EFA282/13 TRANSPRION) , by the INRA 'Institut Carnot en santé animale' and by the ANR grant 'UnmaskingBloodPrions'(ANR-15-CE18-0028).

Biographical Sketch

Jean Yves Douet is assistant lecturer in Ophthalmology at the National Veterinary School of Toulouse and a member of the TSE research group in the UMR INRA ENVT 1225 unit. His primary research interests are the pathogenesis of the prion disease with special emphasis on the iatrogenic risk of transmission.

References

1. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell. 1983;35(1):57-62.
2. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature. 1997;389(6650):498-501.
3. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature. 1996;383(6602):685-90.
4. Garske T, Ghani AC. Uncertainty in the tail of the variant Creutzfeldt-Jakob disease epidemic in the UK. PLoS One. 2010;5(12):e15626.
5. Gill ON, Spencer Y, Richard-Loendt A, Kelly C, Dabaghian R, Boyes L, et al. Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey. BMJ. 2013;347:f5675.
6. Wadsworth JD, Joiner S, Hill AF, Campbell TA, Desbruslais M, Luthert PJ, et al. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet. 2001;358(9277):171-80.
7. Haik S, Faucheux BA, Sazdovitch V, Privat N, Kemeny JL, Perret-Liaudet A, et al. The sympathetic nervous system is involved in variant Creutzfeldt-Jakob disease. Nat Med. 2003 Sep;9(9):1121-3.
8. Head MW, Ritchie D, Smith N, McLoughlin V, Nailon W, Samad S, et al. Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt-Jakob disease: an immunohistochemical, quantitative, and biochemical study. Am J Pathol. 2004 Jan;164(1):143-53.
9. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature. 2001;411(6839):810-3.
10. Moda F, Gambetti P, Notari S, Concha-Marambio L, Catania M, Park KW, et al. Prions in the urine of patients with variant Creutzfeldt-Jakob disease. N Engl J Med. 2014 Aug 7;371(6):530-9.
11. Lacroux C, Comoy E, Moudjou M, Perret-Liaudet A, Lugan S, Litaise C, et al. Preclinical detection of variant CJD and BSE prions in blood. PLoS Pathog. 2014 Jun;10(6):e1004202.
12. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet. 2004 Aug 7;364(9433):527-9.
13. Uro-Coste E, Cassard H, Simon S, Lugan S, Bilheude JM, Perret-Liaudet A, et al. Beyond PrP res type 1/type 2 dichotomy in Creutzfeldt-Jakob disease. PLoS Pathog. 2008 Mar;4(3):e1000029.
14. Moreno CR, Moazami-Goudarzi K, Laurent P, Cazeau G, Andreoletti O, Chadi S, et al. Which PrP haplotypes in a French sheep population are the most susceptible to atypical scrapie? Arch Virol. 2007;152(6):1229-32.
15. Castilla J, Gutierrez Adan A, Brun A, Pintado B, Ramirez MA, Parra B, et al. Early detection of PrPres in BSE-infected bovine PrP transgenic mice. Arch Virol. 2003 Apr;148(4):677-91.
16. Douet JY, Zafar S, Perret-Liaudet A, Lacroux C, Lugan S, Aron N, et al. Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease. Emerg Infect Dis. 2014 Jan;20(1):114-7.
17. Arnold ME, Hawkins SA, Green R, Dexter I, Wells GA. Pathogenesis of experimental bovine spongiform encephalopathy (BSE): estimation of tissue infectivity according to incubation period. Vet Res. 2009 Jan-Feb;40(1):8.

18. Groschup MH, Buschmann A. Rodent models for prion diseases. Vet Res. 2008 Jul-Aug;39(4):32.
19. Douet JY, Lacroux C, Corbiere F, Litaise C, Simmons H, Lugan S, et al. PrP expression level and sensitivity to prion infection. J Virol. 2014 May;88(10):5870-2.
20. Feraudet C, Morel N, Simon S, Volland H, Frobert Y, Creminon C, et al. Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. J Biol Chem. 2005 Mar 25;280(12):11247-58.
21. Langeveld JP, Jacobs JG, Erkens JH, Bossers A, van Zijderveld FG, van Keulen LJ. Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC Vet Res. 2006;2:19.
22. Cassard H, Torres JM, Lacroux C, Douet JY, Benestad SL, Lantier F, et al. Evidence for zoonotic potential of ovine scrapie prions. Nature communications. 2014;5:5821.
23. Lacroux C, Corbiere F, Tabouret G, Lugan S, Costes P, Mathey J, et al. Dynamics and genetics of PrPSc placental accumulation in sheep. J Gen Virol. 2007 Mar;88(Pt 3):1056-61.
24. Andreoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, van Keulen L, et al. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol. 2000;81 Pt 12:3115-26.
25. Foster JD, Parnham DW, Hunter N, Bruce M. Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. J Gen Virol. 2001;82(Pt 10):2319-26.
26. Lacroux C, Vilette D, Fernandez-Borges N, Litaise C, Lugan S, Morel N, et al. Prionemia and leukocyte-platelet-associated infectivity in sheep transmissible spongiform encephalopathy models. J Virol. 2012 Feb;86(4):2056-66.
27. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet. 2000 Sep 16;356(9234):999-1000.
28. Hilton DA. Pathogenesis and prevalence of variant Creutzfeldt-Jakob disease. J Pathol. 2006 Jan;208(2):134-41.
29. Tamguney G, Richt JA, Hamir AN, Greenlee JJ, Miller MW, Wolfe LL, et al. Salivary prions in sheep and deer. Prion. 2012 Jan-Mar;6(1):52-61.
30. Andreoletti O, Simon S, Lacroux C, Morel N, Tabouret G, Chabert A, et al. PrPSc accumulation in myocytes from sheep incubating natural scrapie. Nat Med. 2004 Jun;10(6):591-3.
31. Chianini F, Cosseddu GM, Steele P, Hamilton S, Hawthorn J, Siso S, et al. Correlation between infectivity and disease associated prion protein in the nervous system and selected edible tissues of naturally affected scrapie sheep. PLoS One. 2015;10(3):e0122785.
32. Hadlow WJ, Kennedy RC, Race RE. Natural infection of Suffolk sheep with scrapie virus. J Infect Dis. 1982;146(5):657-64.
33. Garza MC, Monzon M, Marin B, Badiola JJ, Monleon E. Distribution of peripheral PrP(Sc) in sheep with naturally acquired scrapie. PLoS One. 2014;9(5):e97768.
34. Castilla J, Saa P, Soto C. Detection of prions in blood. Nat Med. 2005 Sep;11(9):982-5.
35. Bougard D, Brandel JP, Belondrade M, Beringue V, Segarra C, Fleury H, et al. Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease. Science translational medicine. 2016 Dec 21;8(370):370ra182.
36. Concha-Marambio L, Pritzkow S, Moda F, Tagliavini F, Ironside JW, Schulz PE, et al. Detection of prions in blood from patients with variant Creutzfeldt-Jakob disease. Science translational medicine. 2016 Dec 21;8(370):370ra183.
37. Bishop MT, Diack AB, Ritchie DL, Ironside JW, Will RG, Manson JC. Prion infectivity in the spleen of a PRNP heterozygous individual with subclinical variant Creutzfeldt-Jakob disease. Brain. 2013 Apr;136(Pt 4):1139-45.

38. Siso S, Gonzalez L, Jeffrey M. Neuroinvasion in prion diseases: the roles of ascending neural infection and blood dissemination. Interdiscip Perspect Infect Dis.2010:747892.
39. Brown P, Brandel JP, Sato T, Nakamura Y, MacKenzie J, Will RG, et al. Iatrogenic Creutzfeldt-Jakob disease, final assessment. Emerg Infect Dis. 2012 Jun;18(6):901-7.
40. Flechsig E, Hegyi I, Enari M, Schwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. Mol Med. 2001;7(10):679-84.
41. Taylor D. Inactivation of the BSE agent. C R Biol. 2002 Jan;325(1):75-6.
42. Molesworth A, Yates P, Hewitt PE, Mackenzie J, Ironside JW, Galea G, et al. Investigation of variant Creutzfeldt-Jakob disease implicated organ or tissue transplantation in the United Kingdom. Transplantation. 2014 Sep 15;98(5):585-9.
43. Jayanthi P, Thomas P, Bindhu P, Krishnapillai R. Prion diseases in humans: oral and dental implications. North American journal of medical sciences. 2013 Jul;5(7):399-403.
44. Peden A, McCardle L, Head MW, Love S, Ward HJ, Cousens SN, et al. Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. Haemophilia. 2010 Mar;16(2):296-304.
45. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet. 2004 Feb 7;363(9407):417-21.
46. Lefrere JJ, Hewitt P. From mad cows to sensible blood transfusion: the risk of prion transmission by labile blood components in the United Kingdom and in France. Transfusion. 2009 Apr;49(4):797-812.
47. Edgeworth JA, Farmer M, Sicilia A, Tavares P, Beck J, Campbell T, et al. Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay. Lancet. 2011 Feb 5;377(9764):487-93.
48. Jackson GS, Burk-Rafel J, Edgeworth JA, Sicilia A, Abdilahi S, Korteweg J, et al. A highly specific blood test for vCJD. Blood. 2014 Jan 16;123(3):452-3.

Table 1: Endpoint titration of a reference vCJD sample (10% weight volume frontal cortex homogenate) in mice expressing the bovine PrP (tgBov)

	Transmission in tgBov	
Dilution	**Pos mice**	**Incubation (mean±SD)**
neat	6/6	249±2 days
10^{-1}	6/6	283±15 days
10^{-2}	6/6	316±21 days
10^{-3}	6/6	342±10 days
10^{-4}	6/6	453±66 days
10^{-5}	2/6	479, 495 days*
10^{-6}	1/6	502 days*
10^{-7}	0/6	>700 days

A 10% weight/volume homogenate was prepared using frontal cortex from a clinically affected vCJD patient. Groups of 6 tgBov mice were inoculated intracerebrally with 20µL of serial ten-fold dilutions of this homogenate. Mice were considered positive when abnormal PrP deposition was detected in the brain. Incubation periods are presented as mean +/-SD, except for those marked (*) indicating dilutions in which less than half of mice were scored as positive.

Table 2: Endpoint titration of the PMCA seeding activity in a reference vCJD brain sample

	Reference vCJD 10% brain homogenate dilution series number of positive PMCA reactions								
Amplification round	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**10^{-10}**
1	6/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
2	6/6	6/6	5/6	3/6	0/6	0/6	0/6	0/6	0/6
3	6/6	6/6	6/6	6/6	3/6	0/6	0/6	0/6	0/6
4	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6
5	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6
6	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6

10% weight/volume homogenate was prepared using frontal cortex from a symptomatic vCJD patient (using the same homogenate as in table 1). Samples were serially diluted ten-fold (10^{-2} to 10^{-10}) before being used to seed PMCA reactions. Six individual replicates of each sample dilution were tested. The PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Reactions were then subjected to 6 amplification rounds, each composed of 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). The number of PrP^{res} WB positive replicates corresponding to each round and each dilution are reported.

Table 3: Characteristics of the patients included in the study

Patient Identifier	Diagnosis	Gender	Year of death	Age at death	Disease duration	*PRNP* codon 129	*PRNP* mutations
vCJD-1	vCJD	M	1999	33 years	18 months	MM	No mutations detected
vCJD-2	vCJD	F	2000	17 years	18 months	MM	No mutations detected
vCJD-4	vCJD	M	2000	26 years	10 months	MM	No mutations detected
vCJD-3	vCJD	M	2001	26 years	10 months	MM	No mutations detected
vCJD-A	Asymptomatic vCJD	F	2004	82 years	N/A	MV	No mutations detected
NC-1	Not CJD (tumour, infarction, ischaemia)	F	2005	85 years	N/A	MM	No consent for sequencing
NC-2	Not CJD (Alzheimer's disease, infarction, ischaemia)	F	2010	80 years	N/A	MM	No mutations detected

Table 4: Results of Protein Misfolding Cyclic Amplification reactions seeded with tissue homogenate from vCJD and control patients

	vCJD clinical (Met_{129}/Met_{129})				vCJD preclinical (Met_{129}/Val_{129})	Non vCJD controls (Met_{129}/Met_{129})	
Tissue	**vCJD-1**	**vCJD-2**	**vCJD-3**	**vCJD-4**	**vCJD-A**	**NC-1**	**NC-2**
Frontal cortex	10^{-8}	10^{-8}	10^{-8}	10^{-8}	neg	neg	neg
Pituitary gland	N/A	N/A	N/A	N/A	10^{-2}	neg	neg
Trigeminal ganglia	N/A	N/A	N/A	N/A	neg	neg	neg
Dorsal root ganglia	N/A	N/A	N/A	N/A	neg	neg	neg
Cervical Lymph node	10^{-5}	10^{-4}	10^{-4}	10^{-3}	10^{-4}	NA	NA
Tonsil	10^{-3}	10^{-4}	10^{-6}	10^{-3}	10^{-3}	NA	neg
Appendix	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-2}	neg	neg
Distal Ileum	10^{-3}	10^{-5}	10^{-5}	10^{-2}	10^{-3}	neg	neg
Spleen	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-3}	neg	neg
Thymus	NA	10^{-3}	10^{-2}	10^{-2}	10^{-2}	NA	NA
Lung	10^{-2}	10^{-2}	neg	neg	10^{-3}	neg	neg
Heart	10^{-2}	10^{-2}	neg	neg	neg	neg	neg
Liver	10^{-4}	10^{-2}	10^{-2}	10^{-4}	10^{-2}	neg	neg
Kidney	10^{-2}	10^{-3}	neg	10^{-3}	neg	neg	neg
Salivary gland	10^{-4}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	neg	neg
Pancreas	10^{-2}	neg	10^{-2}	10^{-4}	neg	neg	neg
Thyroid	10^{-2}	neg	10^{-2}	10^{-2}	neg	neg	neg
Adrenal gland	10^{-3}	10^{-3}	10^{-3}	10^{-4}	neg	neg	neg
Bone marrow	10^{-4}	10^{-5}	10^{-3}	10^{-4}	neg	neg	neg
Skeletal muscle	10^{-4}	10^{-2}	neg	N/A	neg	neg	neg
Testis	neg	NA	neg	10^{-3}	N/A	N/A	N/A
Ovary	N/A	10^{-4}	N/A	N/A	N/A	N/A	N/A

PMCA reactions were seeded with ten-fold serial dilutions of vCJD 10% tissues homogenates (10^{-2} to 10^{-9}) that had been collected post mortem from symptomatic vCJD patients (vCJD-1 to vCJD-4) or an asymptomatic vCJD-infected individual (vCJD-A). At least four replicates of each sample dilution were tested in two independent PMCA runs.

vCJD-1 to vCJD-4 were homozygous for methionine at codon 129 of the *PRNP* gene. Patient vCJD-A was heterozygous (methionine/valine) at codon 129 of the *PRNP* gene. The PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Reactions seeded with tissues from two non-vCJD infected control patients (NC-1 and NC-2) were included as negative controls. PMCA reactions were then subjected to 4 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. The PMCA reactions were analysed by Western Blot (WB) for the presence of PK resistant PrP (PrP^{res} - antibody Sha31 epitope YEDRYYRE). The last positive dilution that resulted in a positive WB signal in at least half of the tested replicates after 4 PMCA amplification rounds is reported in the table. N/A denotes not applicable. Neg denotes negative.

Table 5: Bone marrow sample bioassay in bovine PrP expressing mice (tgBov)

case	Transmission in tgBov		Infectious titer (LD_{50}/g) Mean – (CI 95%)
	Positive/inoculated mice	Incubation (mean±SD)	
vCJD-1	5/5	458±37 days	$10^{3.1}$ ($10^{2.6}$-$10^{3.5}$)
vCJD-2	6/6	373±35 days	$10^{4.7}$ ($10^{4.3}$-$10^{5.2}$)
vCJD-3	4/6	504±10 days	$10^{2.3}$ ($10^{1.8}$-$10^{2.7}$)
vCJD-4	6/6	447±91 days	$10^{4.0}$ ($10^{3.4}$-$10^{4.5}$)
PBS control	0/6	>600 days	N/A

10% weight/volume bone marrow homogenates prepared from four symptomatic vCJD patients (see table 3) was inoculated intracerebrally (IC) into 6 tgBov mice (20µL per mouse). One mouse (inoculated with vCJD-1) died within the first few days following IC inoculation. Mice were euthanized when they showed clinical signs of prion infection or after 600 days post inoculation. Mice were considered prion infected when abnormal PrP deposition was detected in brain. Infectious prion titres were estimated using the method developed by Arnold *et al.* (17). The model uses both the probability of survival (attack rate at each dilution) and the individual mouse incubation periods at each dilution to estimate the infectious load. Infectious titres are given as the estimated value and, in parentheses, the lower and upper limits of the 95% confidence intervals.

Figures:

Figure 1: PMCA amplification of vCJD PrP^{res}

PMCA reactions were seeded with a ten-fold serial dilutions of a reference vCJD brain homogenate (10% weight / volume -10^{-2} to 10^{-10} dilution). This homogenate had been endpoint titrated previously by bioassay in bovine PrP expressing mice (tgBov, intracerebral route – $10^{7.7}$ DL_{50}/g).

PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Unseeded reactions were included as specificity control. PMCA reactions were then subjected to 6 rounds of amplification, each comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). A sheep scrapie in sample was included as control on each gel (WB control).

Figure 2: vCJD Protein Misfolding Cyclic Amplification in peripheral tissues

PMCA reactions were seeded with a ten-fold dilution series of vCJD tissues homogenates (10^{-2} to 10^{-9}) that had been collected post mortem from a vCJD-infected patient at clinical stage or preclinical stage of the disease. At least four replicates of each sample dilution were tested in two independent PMCA runs.

Patients (a) vCJD-1, (b) vCJD-2, (c) vCJD-3 and (d) vCJD-4 died with clinical vCJD. These four patients were homozygotes Methionine at codon 129 of the *PRNP* gene. Patient (e) vCJD-A died while at an asymptomatic or preclinical stage of the disease. This patient was heterozygous Methionine/Valine at codon 129 of the *PRNP* gene.

PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Unseeded reactions and reaction seeded with tissues from two non-vCJD infected control patients (NC-1 and NC-2 see table 3) were included as a specificity control. PMCA reactions were then subjected to 4 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} - antibody Sha31 epitope YEDRYYRE).

For each round, the last dilution displaying a positive WB signal in at least half of the tested replicates is indicated on the graph. (○): round 1 - (▽) : round 2 - (△): round 3 - (■): round 4.

Figure 3: PrP^{res} WB detection in PMCA reactions seeded with peripheral tissues

PMCA reactions were seeded with a ten-fold dilution series (10^{-2} to 10^{-9}) of vCJD tissues homogenates that had been collected post mortem from vCJD patients at clinical stage (symptomatic vCJD case 1 to vCJD case 4) or at an asymptomatic or preclinical stage of the disease (vCJD asymp.) (see table 2). Reactions seeded with tissues from two non-vCJD patients (see table 2) and unseeded PMCA reactions were included as specificity controls. Reactions were then subjected to 4 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700.

The PMCA reactions were analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). On each gel (i) a scrapie in sheep isolate and a vCJD reference isolate were used as control (WB cont.).

570 For the seven presented tissues (frontal cortex, appendix, lung, liver, salivary gland, pancreas
571 and bone marrow) the dilution of the tissues homogenates used to seed the PMCA reactions is
572 indicated below the immunoblots.
573
574

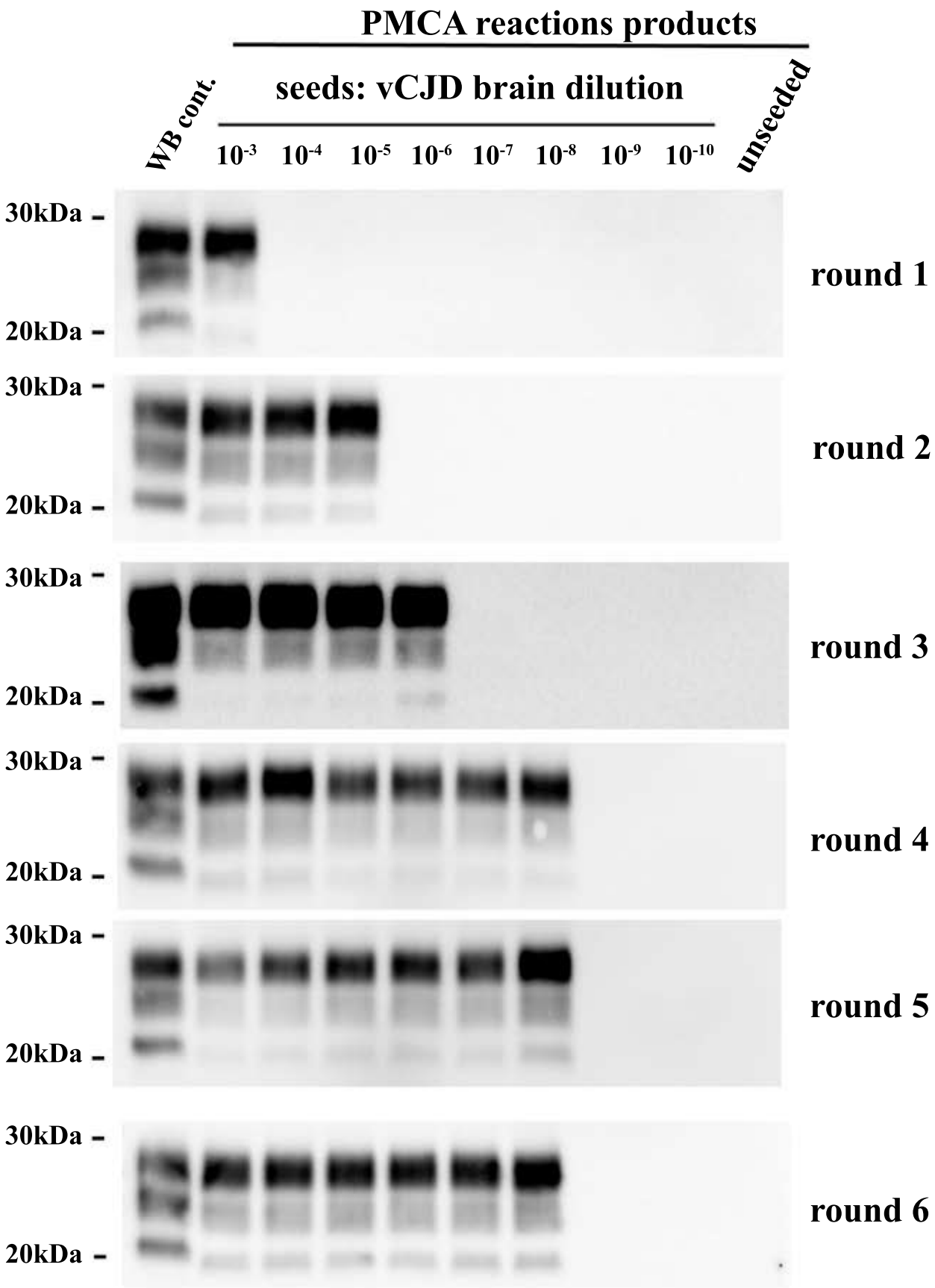
Figure 4: PrP^{res} detection by Western Blot (WB) and Paraffin Embedded Tissue blot (PET blot), in the brain of transgenic mice expressing the bovine-PrP (tgBov).

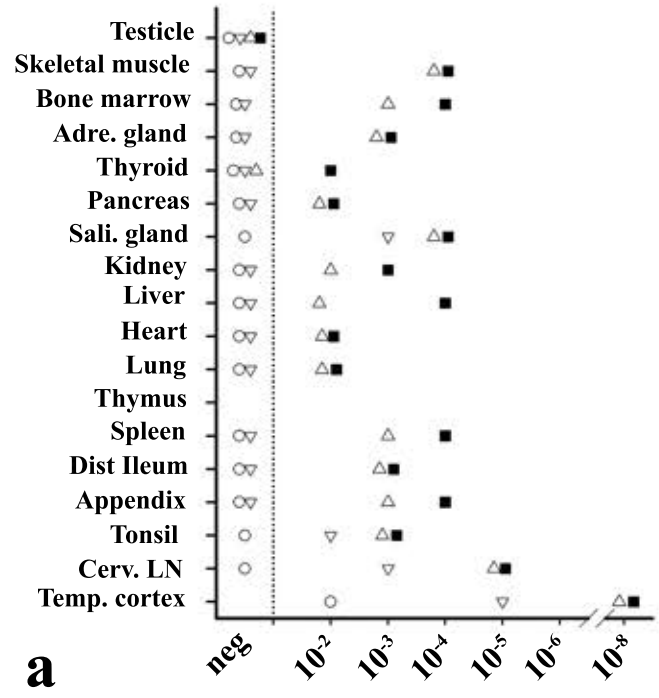
(**a**) PrP^{res} WB of (i) a vCJD sample (frontal cortex) and of tgBov mice (brain) inoculated with (ii) the same vCJD reference isolate or (iii) with bone marrow samples from vCJD affected patients(vCJD 1 to 4 see table 2) and non vCJD control (NC-1, see table 2). On each gel a scrapie isolate (WB cont.) and a non-inoculated tgBov brain homogenate were included as controls. PrP^{res} immunodetection was carried out using Sha31 monoclonal antibody (epitope: $_{145}$YEDRYYRE$_{152}$) and 12B2 epitope (epitope $_{89}$WGQGG$_{93}$).

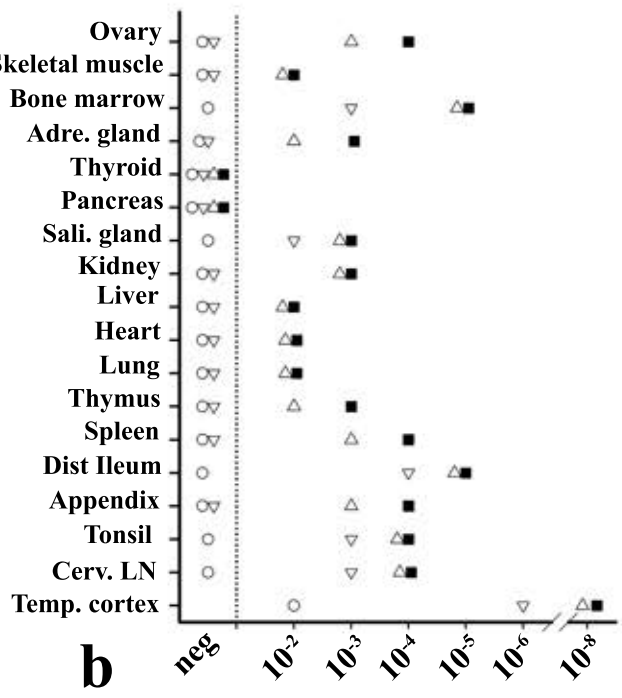
(**b**) PET blot PrP^{res} distribution in coronal section (thalamus level) of tgBov mice inoculated with a reference vCJD isolate (10% brain homogenate) or bone marrow (10% tissue homogenate) from two vCJD patients at clinical stage of the disease (vCJD-1 and vCJD-3 see table 2). PrP^{res} immunodetection was carried out using Sha31 monoclonal antibody (epitope: $_{145}$YEDRYYRE$_{152}$). Bar: 120µm.

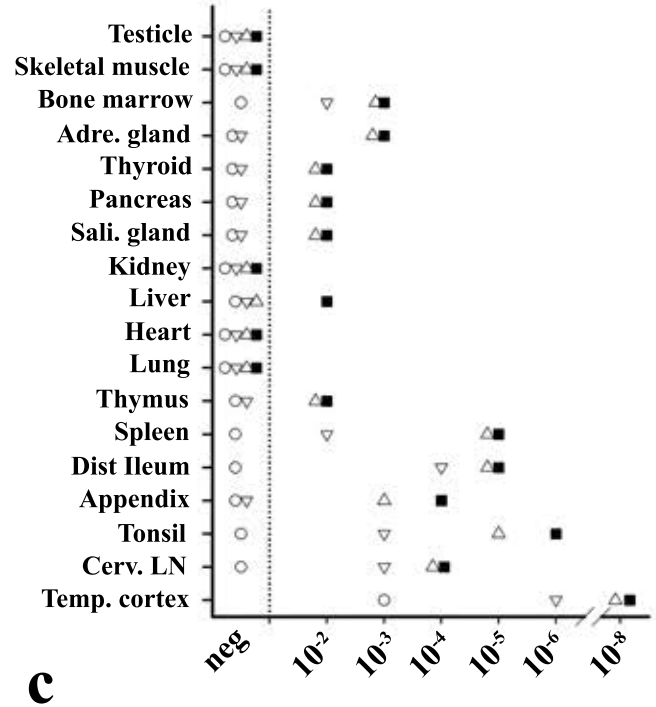
Figure 5: Dose-response relationship for the incubation period and probability of infection of bovine PrP expressing mice.

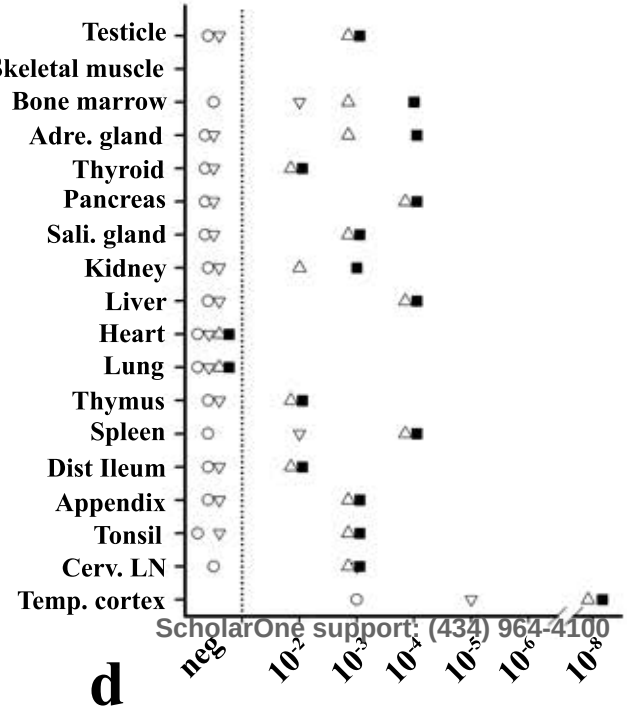
Data derived from an endpoint titration of 10% weight/volume frontal cortex homogenate from a vCJD affected patient in tg Bov mice (20µL intracerebral route, see table 1) were used to establish a model that estimates the infectious titre in an homogenate based on the incubation period and the probability of infection in inoculated mice (model is solid line, observed values given by crosses).

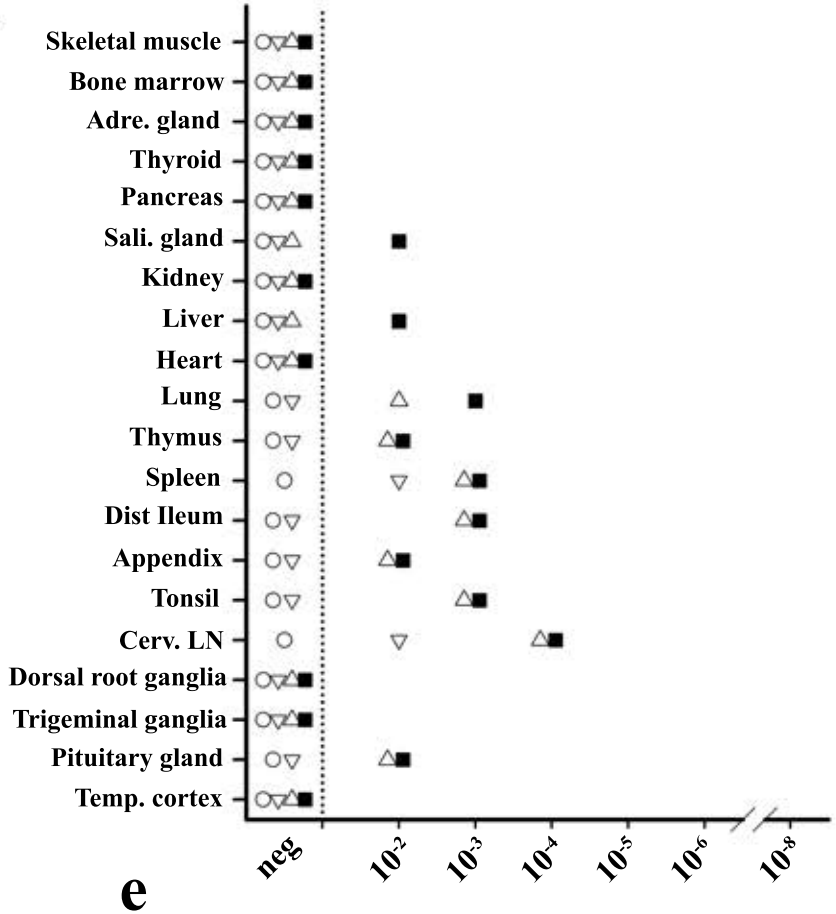

PMCA reactions products
seeds: vCJD brain dilution
WB cont.
10-3
10-4
10-5
10-6
10-7
10-8
10-9
10-10
unseeded
30kDa
20kDa
round 1
round 2
round 3
round 4
round 5
round 6

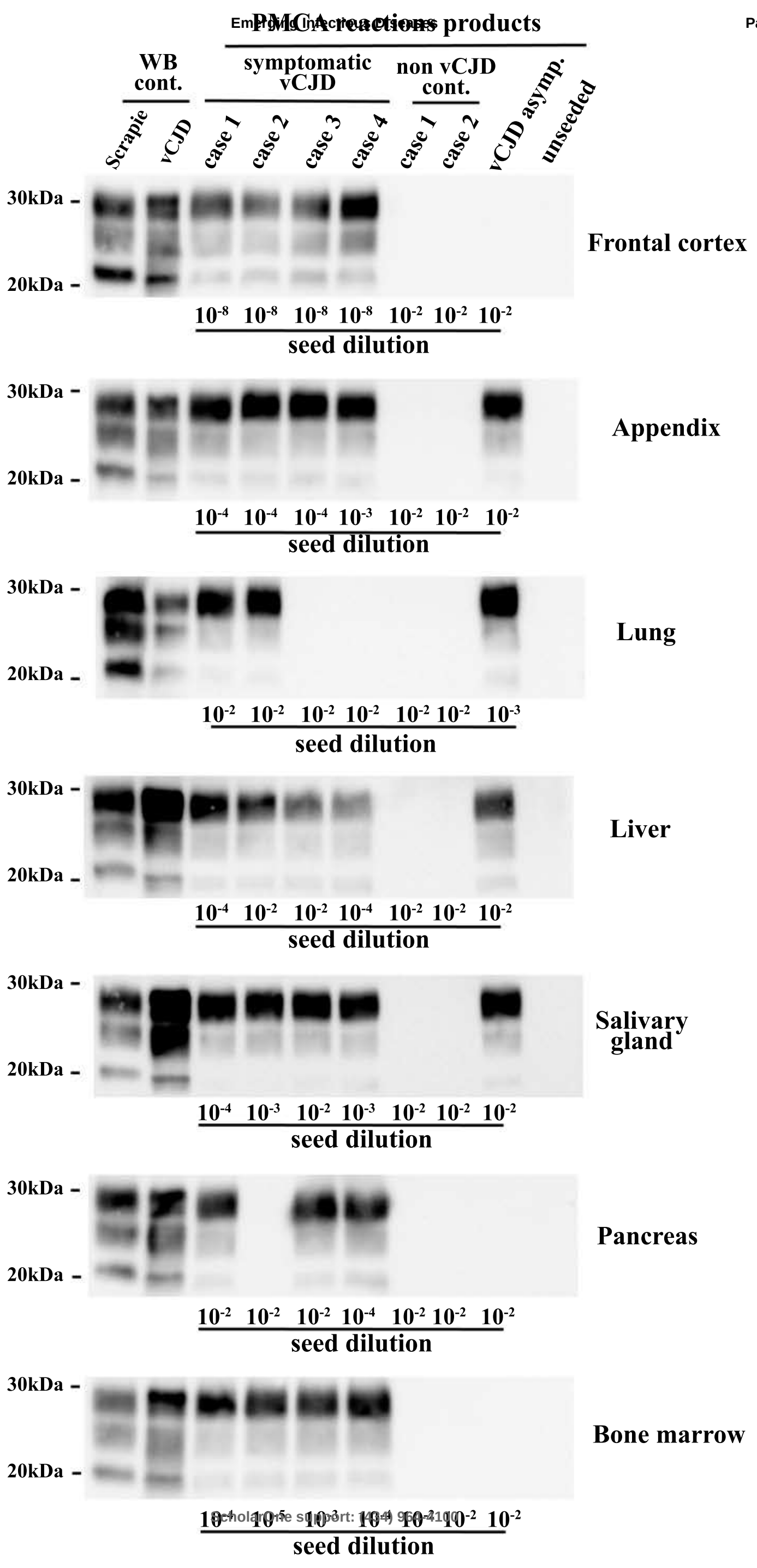


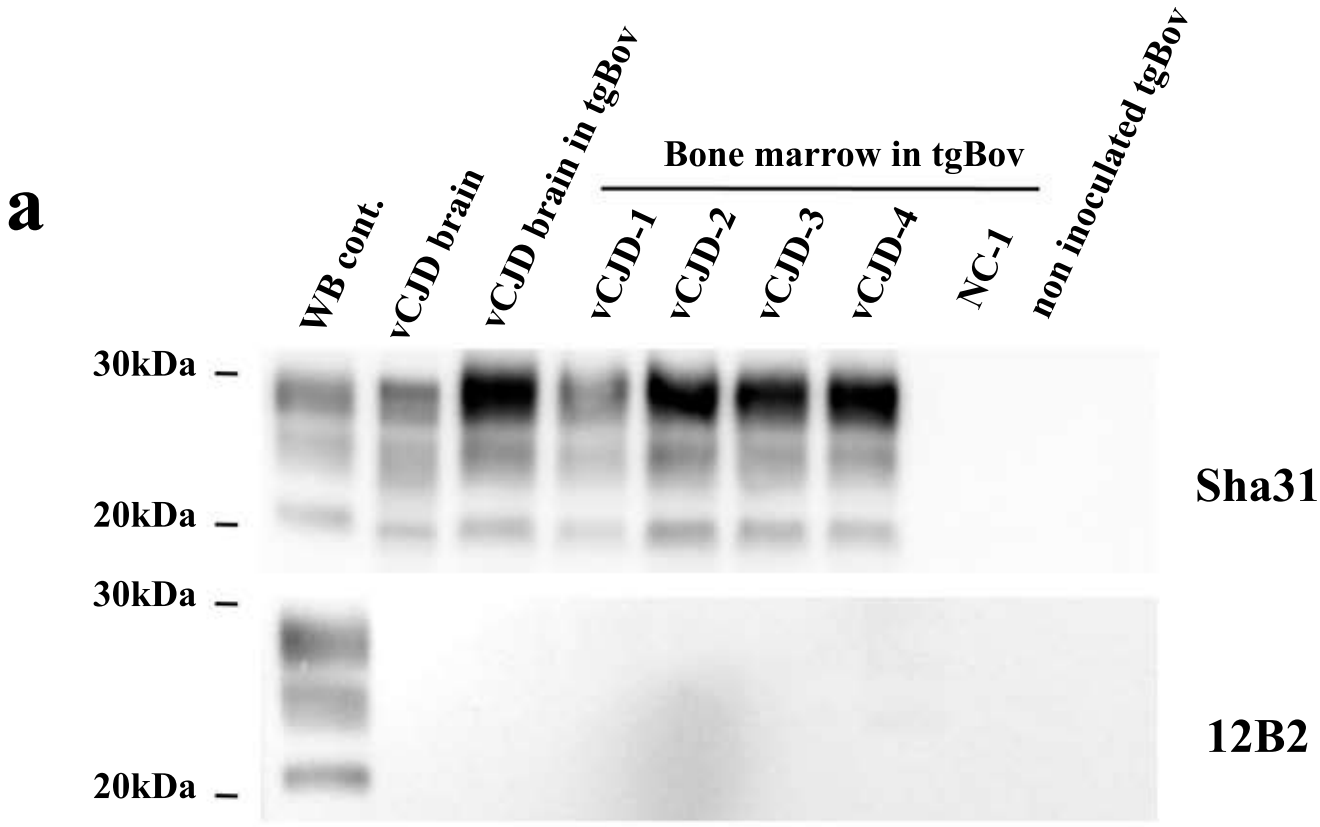


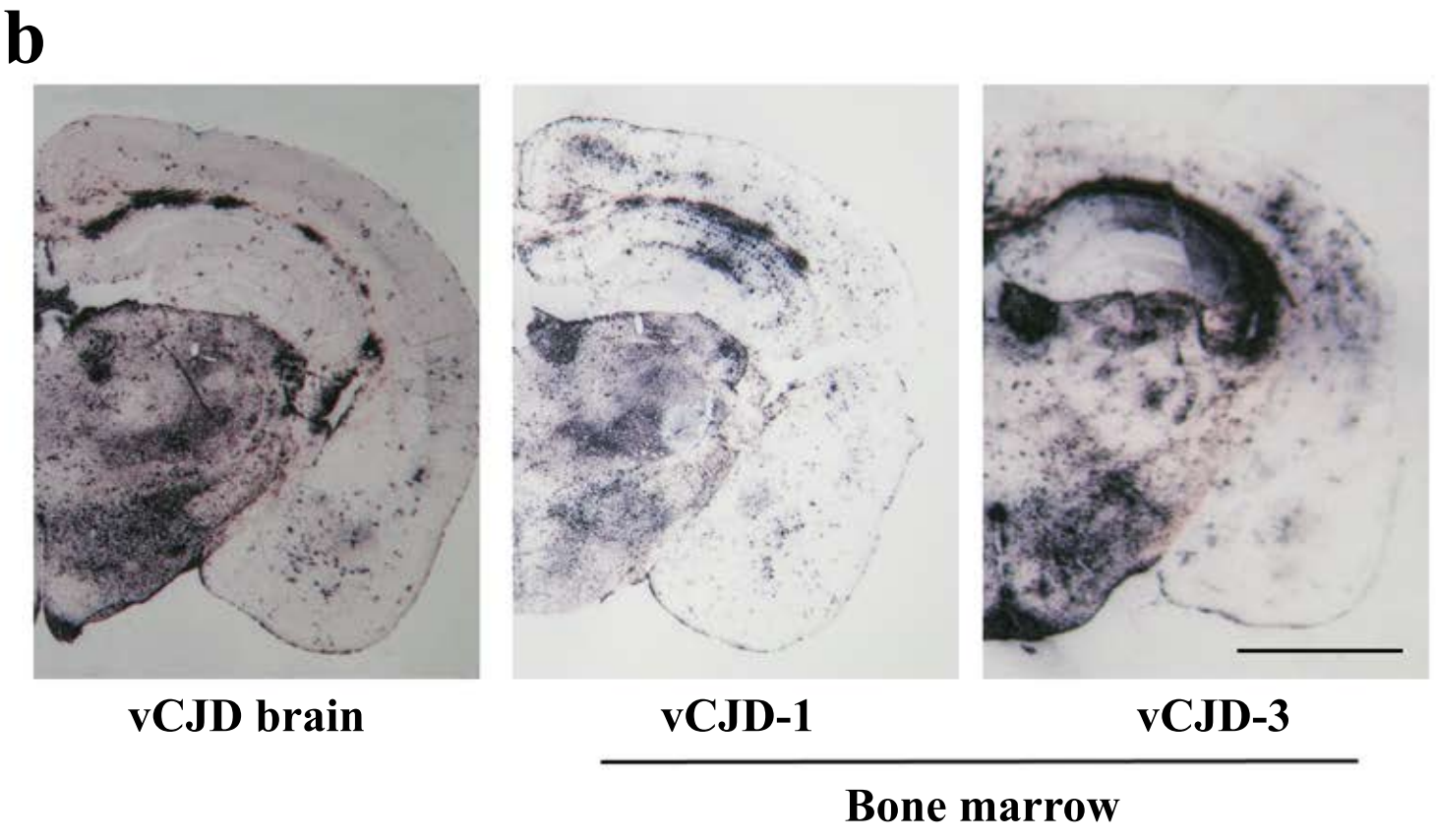


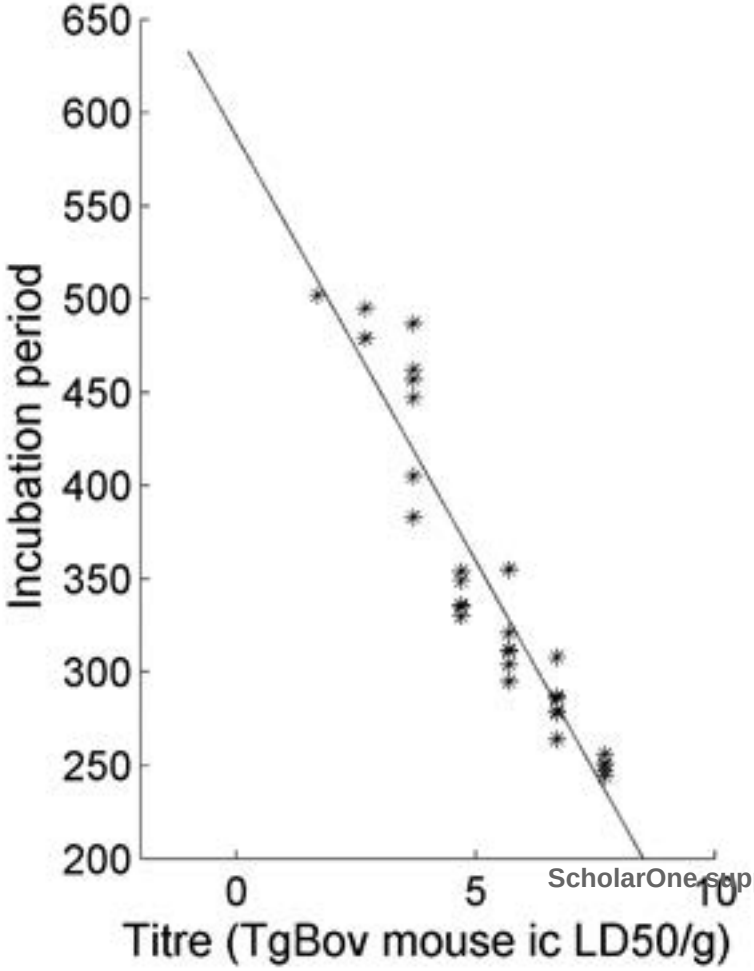


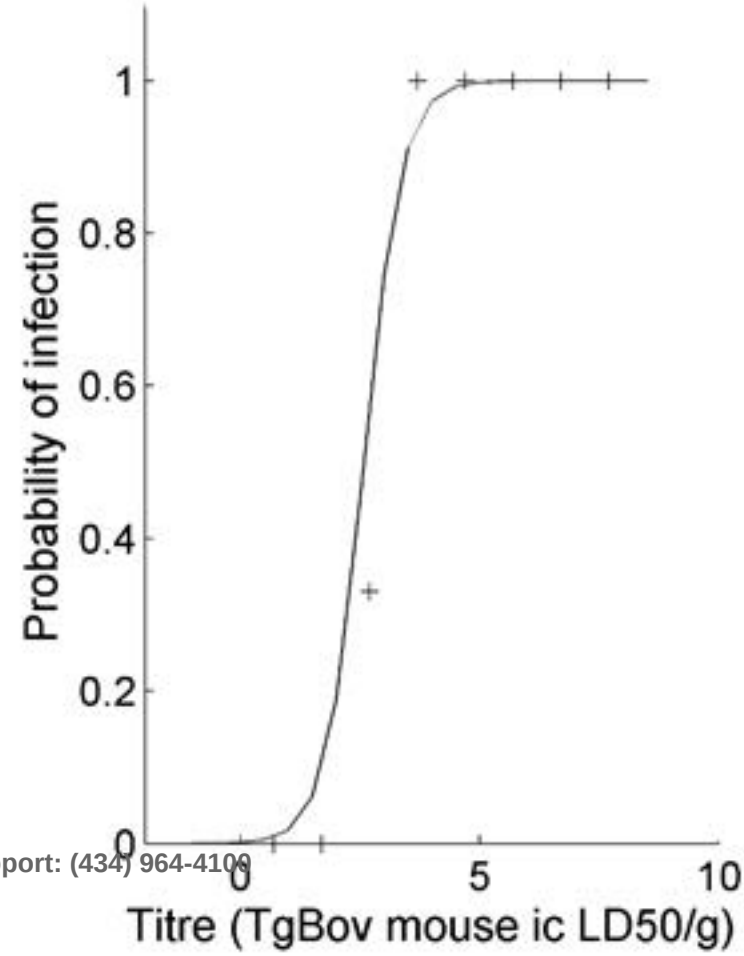





a
WB cont.
vCJD brain
vCJD brain in tgBov
Bone marrow in tgBov
vCJD-1
vCJD-2
vCJD-3
vCJD-4
NC-1
non inoculated tgBov
30kDa
20kDa
30kDa
20kDa
Sha31
12B2


b
vCJD brain
vCJD-1
vCJD-3
Bone marrow





EMERGING INFECTIOUS DISEASES®

Centers for Disease Control and Prevention
1600 Clifton Rd, NE
Mail stop D61
Atlanta, GA 30333
Phone: 404 639-1960
Fax: 404 639-1954

Checklist for Authors: Note that *EID* follows the policies and recommendations of the ICMJE

First Author and Manuscript Title: JY DOUET. **Distribution and quantitative**

Proprietary Considerations	
☑	Authors agree that if their manuscript is accepted for publication in *Emerging Infectious Diseases*, it will upon publication be in the public domain and can be used without liability for copyright infringement.
☑	This manuscript (or one with substantially similar content written by the authors) has not been published and is not being considered for publication elsewhere, including preprint servers.
☑	Corresponding author is the primary contact for reviewing the edited manuscript and galleys.
☑	Financial support for this content is clearly disclosed in the manuscript.
☑	Any organization or person with a financial interest in the subject matter is disclosed in the manuscript.
☑	Authors have disclosed any conflicts of interest related to this article. EID accepts the ICMJE Conflict of Interest form.
☑	This research has been approved by appropriate human or animal subjects research review boards, which are named in the manuscript.
☑	DNA and amino acid sequences have been submitted to an open access sequence database such as GenBank ® and accession numbers are used to refer to the sequences.
☑	Authors have obtained written permission from the publishers of any figures or tables previously published or adapted from published figures or tables.
☑	Authors have obtained written permission from persons identifiable in photographs, case descriptions, or pedigrees.
☑	Authors have obtained written permission from persons named in personal communications (oral or written) stating that they agree to be named and that the information cited is accurate.
Authorship and Acknowledgments	
☑	All authors and contributors meet the ICMJE criteria for those roles, and all persons who made substantial contributions to this work but who did not fulfill the authorship criteria are named in the Acknowledgments.
☑	Written permission has been obtained from all persons listed in the Acknowledgments.
☑	Written permission has been obtained from all persons listed as authors of this manuscript.
☑	A two-sentence biography is provided for the first author or for both authors if there are only two.
Formatting, References, Figures, Word Count, Units of Measure	
☑	Authors have accessed and used guidance provided in the Author Resource Center.
☑	All pages are double-spaced, numbered, and left justified (ragged right margin).
☑	All references are cited in the text in sequential order, comply with Uniform Requirements, and have been checked for accuracy and completeness.
☑	Legends for figures are at the end of the text, following the reference list and any tables.
☑	Each figure or panel of a figure is in a separate file, and each file is at least 300 DPI.
☑	The abstract and manuscript meet specified word counts, which are strictly enforced.
☑	Continuous line numbering is turned on and applied throughout the document.
☑	All units of measure are expressed in SI units.

Additional notes or statements:

Distribution and quantitative estimates of variant Creutzfeldt Jakob Diseases prions in the tissues of clinical and asymptomatic patients

Jean Yves Douet[1], Caroline Lacroux[1], Naima Aron[1], Mark W Head[2], Séverine Lugan[1], Cécile Tillier[1], Alvina Huor[1], Hervé Cassard[1], Mark Arnold[3], Vincent Beringue[4], James W Ironside[2] and Olivier Andréoletti[1 *]

[1] *UMR INRA ENVT 1225, Interactions Hôtes Agents Pathogènes, Ecole Nationale Vétérinaire de Toulouse, 23 Chemin des Capelles 31076 Toulouse, France*

[2] *National Creutzfeldt-Jakob Disease Research & Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh, Western General Hospital, Edinburgh EH42XU, UK*

[3] *APHA Sutton Bonington, Loughborough, Leciestershire, LE12 5NB, UK*

[4] *VIM, INRA, Université Paris-Saclay, 78350, Jouy-en-Josas, France*

Author summary line : A rapid in vitro assay shows that the distribution of vCJD prions in the tissues of clinical and asymptomatic patients is wider than previously thought

Running title: Variant CJD iatrogenic transmission risk

Keywords: Prions, infectivity, public health, vCJD

* Corresponding author: o. andreoletti o.andreoletti@envt.fr

Abstract

In the United Kingdom about 1 in 2000 people could be infected with variant Creutzfeldt-Jakob-Disease (vCJD). In that context, the risk of transmission of vCJD by medical procedures remains a major concern for public health authorities.

In this study, *in vitro* amplification of prions by the Protein Misfolding Cyclic Assay (PMCA) was used to estimate the distribution and the level of vCJD agent in a range of tissues (n=21) from four patients who died with clinical vCJD and one asymptomatic vCJD-infected individual.

Unexpectedly, PMCA demonstrated the presence of significant levels of vCJD prions in numerous tissues, including liver, salivary gland, kidney, lung or bone marrow. Bioassays confirmed that the quantitative estimate of vCJD prion accumulation levels provided by PMCA are indicative of the vCJD infectivity levels in peripheral tissues.

This work provides critical data for the design of rational measures aimed at minimising the risk of iatrogenic transmission of vCJD.

.

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that occur naturally in sheep (scrapie), cattle (bovine spongiform encephalopathy - BSE), and humans (Creutzfeldt-Jakob disease - CJD). A key event in the pathogenesis of TSEs is the conversion of the normal cellular prion protein (PrP^{C}, which is encoded by *PRNP*) into an abnormal disease-associated isoform (PrP^{Sc}) in tissues of infected individuals. PrP^{C} is completely degraded after controlled digestion with proteinase K (PK) in the presence of non-denaturing detergents. PrP^{Sc} is N-terminally truncated under such conditions, leaving a PK resistant core, termed PrP^{res} (1).

In 1996, a new form of CJD, termed variant CJD (vCJD) was identified in the UK and is believed to be due to zoonotic transmission of the agent that causes BSE in cattle, probably as a consequence of dietary exposure to BSE-contaminated meat products (2, 3).

The total number of vCJD clinical cases identified so far remains limited (227 patients worldwide at the time of writing). However, the prevalence of asymptomatic vCJD infection in populations exposed to the BSE agent remains extremely uncertain (4). In the UK 32,441 appendix samples, collected during surgery on patients born between 1941 and 1985 were tested for abnormal prion protein accumulation by immunohistochemistry. This study indicated a likely vCJD prevalence estimate of 1 in 2,000 in these age cohorts (95% Confidence Interval ranging from 1 in 3,500 to 1 in 1,250) (5). Outside the UK, there is no data available concerning the prevalence of asymptomatic vCJD infection in the population, although BSE exposure is likely to have occurred in several continental European counties as judged by cases of vCJD in these counties which have no known link to BSE exposure in the UK (http://www.cjd.ed.ac.uk/documents/worldfigs.pdf)

Over the last two decades a substantial number of studies have focused on the distribution of the vCJD agent in the tissues of infected patients (6-8). Most have failed to demonstrate a

convincing accumulation of the vCJD agent outside the nervous system (central, peripheral and autonomic) and lymphoid tissues. However, the sensitivity of the PrP^{res} detection techniques used in these investigations was limited.

The Protein Misfolding Cyclic Amplification (PMCA) technology is thought to mimic prion replication *in vitro* but in accelerated form, allowing amplification of minute amounts of PrP^{Sc} and prion infectivity (9). It is facilitated by combining a PrP^{C}-containing substrate with a seed comprising a tissue homogenate containing previously undetectable amounts of PrP^{Sc}. By repetitive cycles of incubation and sonication, the levels of PrP^{Sc} in the seed are increased to levels at which they can be detected by conventional biochemical techniques. Recently the capacity of PMCA to detect endogenous vCJD agents present at very low levels in biological fluids such as urine and blood from infected patients has been shown to be possible (10, 11). In this study, we first evaluated the relative sensitivity of the PMCA assay versus bioassay in mice for the detection the vCJD agent. We then estimated, using PMCA, the level of vCJD prions in 21 tissues collected post from patients who died with symptomatic vCJD (n=4) and from a patient with an asymptomatic vCJD infection. Finally we determined whether the vCJD prion levels as estimated by PMCA were consistent with the infectious titres as estimated by bioassay using transgenic mice.

Methods

Ethics Statement

All animal experiments have been performed in compliance with institutional and French national guidelines, in accordance with the European Community Council Directive 86/609/EEC. The animal experiments that are part of this study (national registration 01734.01) were approved by the ENVT ethic committee. Mouse inoculations were performed under anaesthesia (isofulorane). Mice that displayed clinical signs were anesthetized with isofluorane before killing by CO2 inhalation.

Human samples were obtained from the National CJD Research & Surveillance Unit Brain and Tissue Bank in Edinburgh, UK, which is part of the Edinburgh Brain Bank. For the purposes of this study, samples were pseudo-anonymized using a Brain Bank reference number. All UK cases had informed written consent for research and their supply and use in this study was covered by Ethics Approval (LREC 2000/4/157).

Control and vCJD infected patients

Tissues from four clinical vCJD cases (referred to in this study as vCJD-1 to vCJD-4) and one asymptomatic vCJD- infected individual who received a transfusion of packed red blood cells from a donor who subsequently died from vCJD (12) (vCJD-A) were investigated. Tissues from two non-vCJD infected patients (NC-1 and NC-2) were used as control. Information related to the vCJD and control patients are presented in table 3. In cases with appropriate consent, the entire *PRNP* coding sequence was established as to exclude pathogenic mutations in the *PRNP* gene (13, 14). Table 4 indicates the list of tissue tested in each patient.

Mouse bioassays

Bioassay were carried out in mice expressing the bovine PrP (tgBov-Tg100) as previously described (15, 16). These mice were observed daily and their neurological status was assessed weekly. When clinically progressive TSE disease was evident, or at the end of their lifespan, the animals were euthanized. Survival time was expressed as the mean of the survival days post inoculation (dpi) of all the mice scored positive for PrP^{res}, with a corresponding standard deviation. In cages where no clinical signs were observed, mice were killed at the end of their natural life-span (600 to 800 days). In those cases, incubation periods reported in the table (>600 dpi) corresponded to the survival time observed in at least three out of the six mice.

Infectious prion titre estimates

The infectious titre in a reference 10% weight/vol temporal cortex homogenate from a clinical vCJD patient was established by endpoint titration (Intracerebral route) in tgBov mice. The infectious titre (LD_{50}/g IC in tgBov) was estimated by the Spearman's method.

The titre of prion infectivity in vCJD affected patients bone marrow samples was estimated using the method developed by Arnold *et al.* (17). The model uses both the probability of survival (attack rate at each dilution) and the individual mouse incubation periods at each dilution to estimate infectious load and thus is able to provide more accurate estimation of titre than using either attack rate or incubation period data alone.

PMCA reactions

A transgenic mouse line that expresses ovine $A_{136}R_{154}Q_{171}$ PrP variant PrP^{C} (tgShXI) was used to prepare the PMCA substrate as previously described (18, 19). PMCA amplification was performed as previously described (11). Each PMCA run included a reference vCJD sample (10% brain homogenate) as a control for the amplification performance. Unseeded controls (1 unseeded control for 8 seeded reactions) were also included in each run. For each

tested dilution of each sample at least four replicates of each these sample dilutions were tested in two independent run. For each sample the last dilution showing at least 50% of positive replicates (presence of detectable PrP^{res} in the reaction as assessed by WB) was determined.

Abnormal PrP Western blot (WB) detection

PK resistant abnormal PrP extraction (PrP^{res}) and Western blot were performed as previously described (11). Immunodetection was performed using two different monoclonal PrP-specific antibodies, Sha31 (1 µg/ml) (20), and 12B2 (4 µg/ml) (21) which recognize the amino acid sequences YEDRYYRE (145-152), and WGQGG (89-93) respectively.

Paraffin embedded tissue blot

Paraffin embedded tissue was performed as previously described (22, 23).

Results

Sensitivity of vCJD agent detection by PMCA and bioassay

In order to determine the relative sensitivity of PMCA, a reference isolate (10% cerebral cortex homogenate from a vCJD affected patient) that had previously undergone end point titration (IC inoculation route –sup table 1) in bovine PrP expressing mice (tgBov) was re-titrated by PMCA.

The amplification of a 1/10 dilution series of this sample (6 individual replicates per dilution point) demonstrated that four PMCA rounds (24 hours per round *i.e.* 96 hours) were sufficient to reach the maximal sensitivity level of the assay. Additional PMCA rounds improved neither the analytical sensitivity of the assay nor the number of positive replicates (table 2 – figure 1). Based on these results, the seeding activity (SA) of the isolate was estimated by the Spearman's approach to be $10^{10.97}$ SA_{50} per gram. The bioassay end point titration data of the same isolate in tgBov indicated an infectious titre of $10^{7.7}$ LD_{50} per gram. Taking into account the 4 fold lesser amount of material used to seed a PMCA reaction in comparison to the material used in mouse inoculations, these results support the contention that PMCA protocol we used was 465 times more sensitive than bioassay in tgBov mice for the detection of vCJD prions.

PMCA results in control and vCJD affected patients

Based on these results, 1/10 dilutions series of the 10% homogenates were prepared from the tissues collected in vCJD affected and non-vCJD control patients (table 3) before being subjected to four PMCA amplification rounds. After each amplification round, the PMCA reaction products were tested for the presence of PrP^{res} by Western Blot (table 4 and figure 2). None of the reactions seeded with tissues from the non-CJD controls were found to be positive (table 4). In contrast, the PMCA reactions seeded with numerous tissues from the

four symptomatic vCJD patients were found to be positive for PrP^{res} (table 4- figure 2). As expected, among the tested tissues the brain homogenates (temporal cortex) showed the highest seeding activity (last PrP^{res} positive dilution 10^{-8}). All the tested lymphoid organs also displayed seeding activity, but the last tested PMCA positive dilution varied according to the tested organs from 10^{-2} (thymus) to 10^{-6} (distal ileum and tonsil). Moreover, for a given lymphoid organ, up to 10^{2} fold differences were observed in seeding activity depending on the patient and sample tested. Taken together these data indicate that in symptomatic vCJD patients the lymphoid organs contain between 10^{2} and 10^{6} fold less prion seeding activity than the same amount of brain tissue (table 4).

In the four symptomatic vCJD patients PrP^{res} was observed in reactions seeded with salivary gland, adrenal gland, liver and bone marrow (figure 2 and 3). According to the last dilution showing a seeding activity, the vCJD agent in these tissue was 10^{3} to 10^{6} fold lower than in the brain. PrP^{res} was also detected in the PMCA reactions seeded with heart, liver, kidney, skeletal muscle, several endocrine/exocrine glands (pancreas, thyroid) and the gonads, from some, but not all, of the clinical vCJD patients. Where positive, these tissue contained a level of vCJD seeding activity that was equivalent to those observed in distal ileum (*i.e* 10^{3} to 10^{6} fold lower than in the brain).Whichever the tissue used to seed the PMCA reactions, the PrP^{res} western blot profile in positive reactions were indistinguishable from that observed in reactions seeded with the vCJD brain control (figure 3).

Analysis of tissues from an asymptomatic vCJD-infected individual

No prion seeding activity was detected in the temporal cortex of this asymptomatic vCJD infected patient, who was a *PRNP* codon 129 heterozygote (Met/Val_{129}) (12) (table 4, figure 2). PMCA reactions seeded with the dorsal root ganglia or trigeminal ganglia homogenates

198 from this patient were also negative. However, a low seeding activity was detected in the
199 pituitary gland (last PrP^{res} positive dilution 10^{-2} dilution). Additionally, as with the
200 symptomatic vCJD patients, PMCA amplification readily detected the presence of vCJD
201 prions in all the tested lymphoid organs in this asymptomatic individual. According to the
202 PMCA results, the vCJD agent load in lymphoid organs in this *PRNP* codon 129 Met/Val
203 asymptomatic patient were similar to those observed in the Met/Met_{129} patients at the clinical
204 stage of the disease. Beside the lymphoid organs, prion seeding activity was present in certain
205 peripheral tissues from this patient (notably salivary gland, lung and liver) (table 4, figure 2
206 and 3). Strikingly, certain tissues that contained a substantial prion seeding activity in the
207 clinically affected patients (such as bone marrow or the adrenal gland) were found to be
208 negative. Here too, whichever the tissue used to seed the PMCA reactions, the PrP^{res} WB
209 profile observed in positive reactions was similar to the PrP^{res} WB profile in reaction seeded
210 with the control vCJD brain (figure 3).
211
212 **vCJD infectivity in bone marrow**
213 In order to confirm that the presence of PMCA seeding activity in peripheral tissues of vCJD
214 patients correlated to the presence of infectivity, the bone marrow samples from the
215 symptomatic patients used in PMCA experiments were inoculated to tgBov mice. A clinical
216 TSE was observed in mice that were inoculated with the four bone marrow samples. The
217 PrP^{res} western blot profile and the PrP^{res} distribution pattern as assessed by PET Blot in the
218 brain of the bone marrow- inoculated mice were identical to the one observed in tgBov mice
219 inoculated with the vCJD brain control (figure 4).
220 The data collected in mice inoculated with the bone marrow samples were also used to
221 estimate prion infectivity levels in these samples. For this purpose, we applied the method
222 developed by Arnold *et al.* (17). The approach combines the probability of survival (attack

rate) and the individual mouse incubation period to provide an estimation of the infectious titres. Data corresponding to endpoint titration in tgBov mice of the reference vCJD isolate (temporal cortex from clinical vCJD patients) (table 1) were used to derive the relationship between the prion titre of inoculum and the probability of infection and the length of the incubation period (figure 5). According to this model the bone marrow samples displayed an infectious titre that estimated to range between $10^{2.28}$ and $10^{4.72}$ LD_{50}/g in tgBov (table 5). These values are consistent with a 10^3 to 10^5 lower infectivity load in bone marrow samples than in the reference vCJD brain isolate. Interestingly, in keeping with the PMCA results (table 4), the prion load in bone marrow samples (last PrP^{res} positive dilution 10^{-3} to 10^{-5}) were also 10^3 to 10^5 folds lower than in this reference vCJD isolate (last PrP^{res} positive dilution 10^{-8}). Taken together these results strongly support the contention that the PMCA seeding activity provides a reliable estimate of the prion load in the tissues of vCJD-infected patients.

Discussion

While most of the previous investigations carried out on tissue from vCJD patients failed to identify consistent accumulation of the vCJD agent outside the nervous and the lympho-reticular systems, the data obtained in this study demonstrate the presence of vCJD prions in a wide and unexpected variety of peripheral tissues.

Natural scrapie and experimental BSE in sheep are two models of orally transmitted prion diseases (24, 25). In both diseases, the agent accumulates in the lympho-reticular system and in the enteric nervous system during the early preclinical phase of the incubation period. Moreover, an early and persistent prionemia is observed in infected but asymptomatic animals (26, 27). These features were also observed in human vCJD and together with the origin of vCJD (oral exposure to BSE agent), these similarities progressively led to the consensus that BSE/scrapie in sheep and vCJD in human share a common pathogenesis (28).

While the presence of vCJD prions in a variety tissues like bone marrow, kidney, salivary gland, the skeletal muscle, the pancreas, the liver or the heart could seem surprising at first glance, each of these tissue was demonstrated to accumulate prion infectivity and/or abnormal prion protein in TSE infected sheep (29-33).

The *PRNP* codon 129 Met/Val case included in this study is one of only two asymptomatic vCJD-infected individuals who died before the onset of any clinical symptoms of vCJD, and the only case with consent to sample autopsy tissues for research. In this patient, all previous investigations failed to reveal the presence of abnormal prion protein or infectivity in the brain (12, 34). The results we obtained using the cerebral cortex, the dorsal root ganglia and the trigeminal ganglia of this patient, as PMCA seeds are consistent with a lack of CNS involvement by the vCJD agent at the time of death. However, the presence of PMCA seeding activity in the pituitary gland is perhaps surprising in this context.

The presence of abnormal prion protein accumulation in the pituitary gland and other circumventricular organs prior to deposition of PrP^{res} in surrounding brain is a phenomenon that has been reported in TSE infected sheep(35). However, this phenomenon in animals does not seem to represent the main route for neuro-invasion and it is a likely consequence of hematogenous dissemination of the TSE agent through the fenestrated capillary system of the circumventricular organs that is substantially more permeable than the other capillaries in the brain (the "blood-brain barrier"). This finding may therefore be a consequence of the likely haematogenous route of vCJD infection in this individual, via transfusion of packed red blood cells from a vCJD-infected donor, in contrast to a likely oral route of infection in the clinical vCJD cases. (12)

vCJD prions were detected in some peripheral tissues that had been collected in this *PRNP* codon 129 Met/Val patient. However, if the distribution of the vCJD seeding activity in the lymphoreticular tissues was similar to the one observed in the symptomatic vCJD patients, a number of tissues that had been found positive in clinically affected patients were negative in this patient. These findings suggest that the involvement of certain peripheral tissues might occur at a later stage in the incubation period than the one reached by this patient and/or could involve a recirculation of the agent from the CNS (*i.e* centrifugal spread at a late state). However, we cannot discount the possibility that that these differences in tissue distribution are due to either the likely haematogenous route of infection in this individual (as opposed to the likely oral route in the clinical vCJD cases) or the difference between the *PRNP* codon 129 genotype of the asymptomatic vCJD infected individual (*PRNP* codon 129 Met/Val) and the clinical vCJD cases (*PRNP* codon 129 Met/Met)

Irrespective of the explanation for these differences, the presence of vCJD agent in peripheral tissues of patients at both preclinical and clinical stage of the disease raises the potential iatrogenic transmission of this fatal neurological condition by medical procedures. Furthermore, it shows that for certain peripheral tissues end stage titre (and attendant risk) is reached at a pre-clinical stage.

Several hundred iatrogenic cases of CJD have been reported worldwide. These cases appear to result from the transmission of sporadic CJD (sCJD) and most cases have occurred in the recipients of human dura mater grafts or the administration of human growth hormone extracted from cadaveric pituitaries (36). While in sCJD the distribution of the agent is mainly restricted to the nervous system (central and peripheral), the wide distribution of the vCJD agent in asymptomatic but infected patient we described increases the potential number of medical procedures that might result in the iatrogenic transmission of vCJD (dentistry, organ transplant, surgery involving non-disposable equipment) (37-40).

Nevertheless, more than 20 years after the identification of the first vCJD patient, only five cases that are a likely consequence of an iatrogenic vCJD transmission have come to light, all in the UK. These cases were due to either the transfusion of non-leukodepleted red cell concentrates or the treatment with large volumes of pooled UK plasma that included donations from individuals who later developed vCJD (12, 41-43). None of the 220 other vCJD cases identified worldwide have been linked to any other medical or dental procedure.

This limited number of vCJD secondary cases identified so far is certainly reassuring. However, it would be unwise to disregard the threat that vCJD still represents for public health. Despite the relatively low number of vCJD clinical cases observed so far (n=178) in the United Kingdom, the most recent epidemiological studies indicate that, 1 out 2000 people in the UK could carry the vCJD agent (as judged by the presence of abnormal prion protein

detected by immunohistochemistry in lymphoid follicles in the appendix). Each asymptomatic vCJD-infected person represents a potential source of secondary transmission of the disease. In that context, the data we reported here offer an opportunity for refining the measures that were implemented in many countries to limit the risk of vCJD iatrogenic transmission occurrence. The apparent concordance of PMCA biochemical and infectivity bioassay data, and the higher analytical sensitivity of the PMCA assay suggest that future research in this area need not rely exclusively on time-consuming and costly animal bioassay.

Our results clearly highlight the need for vCJD screening assays. After more than a decade of effort several vCJD blood detection tests have reached a stage in their development that could permit their evaluation as screening or confirmatory assays (11, 44, 45). Beyond the ethical issues that the application of a vCJD screening test would represent (in the absence of any treatment and the difficulty to confirm a positive test by another methodology in reasonable time), such assays probably represent the most rational solution for addressing vCJD iatrogenic transmission risks.

Acknowledgments

The National CJD Research & Surveillance Unit is supported by the Policy Research Program of the Department of Health and the Scottish Government (DH121/5061). This report is independent research in part funded by the Department of Health Policy Research Programme and the Scottish Government. The views expressed in this publication are those of the author(s) and not necessarily those of the Department of Health or the Scottish Government. The Edinburgh Brain Bank is supported by the Medical Research Council (MRC G0900580).

Biographical Sketch

Jean Yves Douet is assistant lecturer in Ophthalmology at the National Veterinary School of Toulouse and member of the TSE research group in the UMR INRA ENVT 1225 unit. His primary research interests are the pathogenesis of the prion disease with special emphasis on the iatrogenic risk of transmission.

References

1. McKinley MP, Bolton DC, Prusiner SB. A protease-resistant protein is a structural component of the scrapie prion. Cell. 1983;35(1):57-62.
2. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature. 1997;389(6650):498-501.
3. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature. 1996;383(6602):685-90.
4. Garske T, Ghani AC. Uncertainty in the tail of the variant Creutzfeldt-Jakob disease epidemic in the UK. PLoS One. 2010;5(12):e15626.
5. Gill ON, Spencer Y, Richard-Loendt A, Kelly C, Dabaghian R, Boyes L, et al. Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey. BMJ. 2013;347:f5675.
6. Wadsworth JD, Joiner S, Hill AF, Campbell TA, Desbruslais M, Luthert PJ, et al. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet. 2001;358(9277):171-80.
7. Haik S, Faucheux BA, Sazdovitch V, Privat N, Kemeny JL, Perret-Liaudet A, et al. The sympathetic nervous system is involved in variant Creutzfeldt-Jakob disease. Nat Med. 2003 Sep;9(9):1121-3.
8. Head MW, Ritchie D, Smith N, McLoughlin V, Nailon W, Samad S, et al. Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt-Jakob disease: an immunohistochemical, quantitative, and biochemical study. Am J Pathol. 2004 Jan;164(1):143-53.
9. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature. 2001;411(6839):810-3.
10. Moda F, Gambetti P, Notari S, Concha-Marambio L, Catania M, Park KW, et al. Prions in the urine of patients with variant Creutzfeldt-Jakob disease. N Engl J Med. 2014 Aug 7;371(6):530-9.
11. Lacroux C, Comoy E, Moudjou M, Perret-Liaudet A, Lugan S, Litaise C, et al. Preclinical detection of variant CJD and BSE prions in blood. PLoS Pathog. 2014 Jun;10(6):e1004202.
12. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet. 2004 Aug 7;364(9433):527-9.
13. Uro-Coste E, Cassard H, Simon S, Lugan S, Bilheude JM, Perret-Liaudet A, et al. Beyond PrP res type 1/type 2 dichotomy in Creutzfeldt-Jakob disease. PLoS Pathog. 2008 Mar;4(3):e1000029.
14. Moreno CR, Moazami-Goudarzi K, Laurent P, Cazeau G, Andreoletti O, Chadi S, et al. Which PrP haplotypes in a French sheep population are the most susceptible to atypical scrapie? Arch Virol. 2007;152(6):1229-32.
15. Castilla J, Gutierrez Adan A, Brun A, Pintado B, Ramirez MA, Parra B, et al. Early detection of PrPres in BSE-infected bovine PrP transgenic mice. Arch Virol. 2003 Apr;148(4):677-91.
16. Douet JY, Zafar S, Perret-Liaudet A, Lacroux C, Lugan S, Aron N, et al. Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease. Emerg Infect Dis. 2014 Jan;20(1):114-7.
17. Arnold ME, Hawkins SA, Green R, Dexter I, Wells GA. Pathogenesis of experimental bovine spongiform encephalopathy (BSE): estimation of tissue infectivity according to incubation period. Vet Res. 2009 Jan-Feb;40(1):8.

18. Groschup MH, Buschmann A. Rodent models for prion diseases. Vet Res. 2008 Jul-Aug;39(4):32.
19. Douet JY, Lacroux C, Corbiere F, Litaise C, Simmons H, Lugan S, et al. PrP expression level and sensitivity to prion infection. J Virol. 2014 May;88(10):5870-2.
20. Feraudet C, Morel N, Simon S, Volland H, Frobert Y, Creminon C, et al. Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. J Biol Chem. 2005 Mar 25;280(12):11247-58.
21. Langeveld JP, Jacobs JG, Erkens JH, Bossers A, van Zijderveld FG, van Keulen LJ. Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC Vet Res. 2006;2:19.
22. Cassard H, Torres JM, Lacroux C, Douet JY, Benestad SL, Lantier F, et al. Evidence for zoonotic potential of ovine scrapie prions. Nature communications. 2014;5:5821.
23. Lacroux C, Corbiere F, Tabouret G, Lugan S, Costes P, Mathey J, et al. Dynamics and genetics of PrPSc placental accumulation in sheep. J Gen Virol. 2007 Mar;88(Pt 3):1056-61.
24. Andreoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, van Keulen L, et al. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol. 2000;81 Pt 12:3115-26.
25. Foster JD, Parnham DW, Hunter N, Bruce M. Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. J Gen Virol. 2001;82(Pt 10):2319-26.
26. Lacroux C, Vilette D, Fernandez-Borges N, Litaise C, Lugan S, Morel N, et al. Prionemia and leukocyte-platelet-associated infectivity in sheep transmissible spongiform encephalopathy models. J Virol. 2012 Feb;86(4):2056-66.
27. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. Transmission of BSE by blood transfusion in sheep. Lancet. 2000 Sep 16;356(9234):999-1000.
28. Hilton DA. Pathogenesis and prevalence of variant Creutzfeldt-Jakob disease. J Pathol. 2006 Jan;208(2):134-41.
29. Tamguney G, Richt JA, Hamir AN, Greenlee JJ, Miller MW, Wolfe LL, et al. Salivary prions in sheep and deer. Prion. 2012 Jan-Mar;6(1):52-61.
30. Andreoletti O, Simon S, Lacroux C, Morel N, Tabouret G, Chabert A, et al. PrPSc accumulation in myocytes from sheep incubating natural scrapie. Nat Med. 2004 Jun;10(6):591-3.
31. Chianini F, Cosseddu GM, Steele P, Hamilton S, Hawthorn J, Siso S, et al. Correlation between infectivity and disease associated prion protein in the nervous system and selected edible tissues of naturally affected scrapie sheep. PLoS One. 2015;10(3):e0122785.
32. Hadlow WJ, Kennedy RC, Race RE. Natural infection of Suffolk sheep with scrapie virus. J Infect Dis. 1982;146(5):657-64.
33. Garza MC, Monzon M, Marin B, Badiola JJ, Monleon E. Distribution of peripheral PrP(Sc) in sheep with naturally acquired scrapie. PLoS One. 2014;9(5):e97768.
34. Bishop MT, Diack AB, Ritchie DL, Ironside JW, Will RG, Manson JC. Prion infectivity in the spleen of a PRNP heterozygous individual with subclinical variant Creutzfeldt-Jakob disease. Brain. 2013 Apr;136(Pt 4):1139-45.
35. Siso S, Gonzalez L, Jeffrey M. Neuroinvasion in prion diseases: the roles of ascending neural infection and blood dissemination. Interdiscip Perspect Infect Dis.2010:747892.
36. Brown P, Brandel JP, Sato T, Nakamura Y, MacKenzie J, Will RG, et al. Iatrogenic Creutzfeldt-Jakob disease, final assessment. Emerg Infect Dis. 2012 Jun;18(6):901-7.
37. Flechsig E, Hegyi I, Enari M, Schwarz P, Collinge J, Weissmann C. Transmission of scrapie by steel-surface-bound prions. Mol Med. 2001;7(10):679-84.
38. Taylor D. Inactivation of the BSE agent. C R Biol. 2002 Jan;325(1):75-6.

438 39. Molesworth A, Yates P, Hewitt PE, Mackenzie J, Ironside JW, Galea G, et al.
439 Investigation of variant Creutzfeldt-Jakob disease implicated organ or tissue transplantation in
440 the United Kingdom. Transplantation. 2014 Sep 15;98(5):585-9.
441 40. Jayanthi P, Thomas P, Bindhu P, Krishnapillai R. Prion diseases in humans: oral and
442 dental implications. North American journal of medical sciences. 2013 Jul;5(7):399-403.
443 41. Peden A, McCardle L, Head MW, Love S, Ward HJ, Cousens SN, et al. Variant CJD
444 infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia.
445 Haemophilia. 2010 Mar;16(2):296-304.
446 42. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, et al. Possible
447 transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet. 2004 Feb
448 7;363(9407):417-21.
449 43. Lefrere JJ, Hewitt P. From mad cows to sensible blood transfusion: the risk of prion
450 transmission by labile blood components in the United Kingdom and in France. Transfusion.
451 2009 Apr;49(4):797-812.
452 44. Edgeworth JA, Farmer M, Sicilia A, Tavares P, Beck J, Campbell T, et al. Detection
453 of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay. Lancet. 2011 Feb
454 5;377(9764):487-93.
455 45. Jackson GS, Burk-Rafel J, Edgeworth JA, Sicilia A, Abdilahi S, Korteweg J, et al. A
456 highly specific blood test for vCJD. Blood. 2014 Jan 16;123(3):452-3.
457

458

Table 1: Endpoint titration of a reference vCJD isolate (10% weight volume frontal cortex homogenate) in mice expressing the bovine PrP (tgBov)

	Transmission in tgBov	
Dilution	**Pos mice**	**Incubation (mean±SD)**
neat	6/6	249±2 days
10^{-1}	6/6	283±15 days
10^{-2}	6/6	316±21 days
10^{-3}	6/6	342±10 days
10^{-4}	6/6	453±66 days
10^{-5}	2/6	479, 495 days*
10^{-6}	1/6	502 days*
10^{-7}	0/6	>700 days

A 10% weight/volume homogenate was prepared using frontal cortex from a clinically affected vCJD patient. Groups of 6 tgBov mice were intracerebrally inoculated with 20µL of successive 1/10 dilutions of this homogenate. Mice were considered positive when abnormal PrP deposition was detected in the brain. Incubation periods are presented as mean +/-SD, except for those marked (*) indicating dilutions in which less than half of mice were scored as positive.

Table 2: Endpoint titration of the PMCA seeding activity in a reference vCJD isolate

Amplification round	Reference vCJD 10% brain homogenate dilution series number of positive PMCA reactions								
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
1	6/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
2	6/6	6/6	5/6	3/6	0/6	0/6	0/6	0/6	0/6
3	6/6	6/6	6/6	6/6	3/6	0/6	0/6	0/6	0/6
4	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6
5	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6
6	6/6	6/6	6/6	6/6	6/6	5/6	2/6	0/6	0/6

10% weight/volume homogenate was prepared using frontal cortex from a symptomatic vCJD patient (using the same homogenate as in table 1). Samples were serially 1/10 diluted (10^{-2} to 10^{-10}) before being used for seeding PMCA reactions. Six individual replicates of each sample dilution were tested. The PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Reactions were then subjected to 6 amplification rounds, each composed of 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). The number of PrP^{res} WB positive replicates corresponding to each round and each dilution are reported.

Table 3: Characteristics of the patients included in the study

Patient Identifiant	Diagnosis	Gender	Year of death	Age at death	Disease duration	*PRNP* codon 129	*PRNP* mutations
vCJD-1	vCJD	M	1999	33 years	18 months	MM	No mutations detected
vCJD-2	vCJD	F	2000	17 years	18 months	MM	No mutations detected
vCJD-4	vCJD	M	2000	26 years	10 months	MM	No mutations detected
vCJD-3	vCJD	M	2001	26 years	10 months	MM	No mutations detected
vCJD-A	Asymptomatic vCJD	F	2004	82 years	N/A	MV	No mutations detected
NC-1	Not CJD (tumour, infarction, ischaemia)	F	2005	85 years	N/A	MM	No consent for sequencing
NC-2	Not CJD (Alzheimer's disease, infarction, ischaemia)	F	2010	80 years	N/A	MM	No mutations detected

Table 4: Results of Protein Misfolding Cyclic Amplification reactions seeded with tissue homogenate from vCJD infected and control patients

Tissue	vCJD clinical (Met_{129}/Met_{129})				vCJD preclinical (Met_{129}/Val_{129})	Non vCJD controls (Met_{129}/Met_{129})	
	vCJD-1	vCJD-2	vCJD-3	vCJD-4	vCJD-A	NC-1	NC-2
Frontal cortex	10^{-8}	10^{-8}	10^{-8}	10^{-8}	neg	neg	neg
Pituitary gland	N/A	N/A	N/A	N/A	10^{-2}	neg	neg
Trigeminal ganglia	N/A	N/A	N/A	N/A	neg	neg	neg
Dorsal root ganglia	N/A	N/A	N/A	N/A	neg	neg	neg
Cervical Lymph node	10^{-5}	10^{-4}	10^{-4}	10^{-3}	10^{-4}	NA	NA
Tonsil	10^{-3}	10^{-4}	10^{-6}	10^{-3}	10^{-3}	NA	neg
Appendix	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-2}	neg	neg
Distal Ileum	10^{-3}	10^{-5}	10^{-5}	10^{-2}	10^{-3}	neg	neg
Spleen	10^{-4}	10^{-4}	10^{-5}	10^{-4}	10^{-3}	neg	neg
Thymus	NA	10^{-3}	10^{-2}	10^{-2}	10^{-2}	NA	NA
Lung	10^{-2}	10^{-2}	neg	neg	10^{-3}	neg	neg
Heart	10^{-2}	10^{-2}	neg	neg	neg	neg	neg
Liver	10^{-4}	10^{-2}	10^{-2}	10^{-4}	10^{-2}	neg	neg
Kidney	10^{-2}	10^{-3}	neg	10^{-3}	neg	neg	neg
Salivary gland	10^{-4}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	neg	neg
Pancreas	10^{-2}	neg	10^{-2}	10^{-4}	neg	neg	neg
Thyroid	10^{-2}	neg	10^{-2}	10^{-2}	neg	neg	neg
Adrenal gland	10^{-3}	10^{-3}	10^{-3}	10^{-4}	neg	neg	neg
Bone marrow	10^{-4}	10^{-5}	10^{-3}	10^{-4}	neg	neg	neg
Skeletal muscle	10^{-4}	10^{-2}	neg	N/A	neg	neg	neg
Testis	neg	NA	neg	10^{-3}	N/A	N/A	N/A
Ovary	N/A	10^{-4}	N/A	N/A	N/A	N/A	N/A

PMCA reactions were seeded with ten-fold serial dilutions of vCJD 10% tissues homogenates (10^{-2} to 10^{-9}) that had been collected post mortem in symptomatic vCJD patients (vCJD-1 to vCJD-4) or an asymptomatic vCJD-infected individual (vCJD-A). At least four replicates of each sample dilution were tested in two independent PMCA runs.

vCJD-1 to vCJD-4were homozygous for methionine at codon 129 of the *PRNP* gene. Patient vCJD-A was heterozygous (methionine/valine) at codon 129 of the *PRNP* gene. The PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Reactions seeded with tissues from two non-vCJD infected control patients (NC-1 and NC-2) were included as controls. PMCA reactions were then subjected to 4 amplification rounds each comprising 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. The PMCA reactions were analysed by Western Blot (WB) for the presence of PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). The last positive dilution that resulted in a positive WB signal in at least half of the tested replicates after 4 PMCA amplification rounds is reported in the table. N/A denotes not applicable. Neg denotes negative.

Table 5: Bone marrow sample bioassay in bovine PrP expressing mice (tgBov)

case	Transmission in tgBov		Infectious titer (LD_{50}/g) Mean – (CI 95%)
	Positive/inoculated mice	Incubation (mean±SD)	
vCJD-1	5/5	458±37 days	$10^{3.05}$ ($10^{2.62}$-$10^{3.49}$)
vCJD-2	6/6	373±35 days	$10^{4.72}$ ($10^{4.26}$-$10^{5.18}$)
vCJD-3	4/6	504±10 days	$10^{2.28}$ ($10^{1.83}$-$10^{2.71}$)
vCJD-4	6/6	447±91 days	$10^{3.98}$ ($10^{3.44}$-$10^{4.54}$)
PBS control	0/6	>600 days	N/A

Bone marrow from four symptomatic vCJD patients (see table 3) was inoculated intracerebrally (IC) into 6 tgBov mice (20µL per mouse). One mouse (inoculated with vCJD-1) died within the first few days following IC inoculation. Mice were euthanized when they showed clinical signs of prion infection or after 600 days post inoculation. Mice were considered prion infected when abnormal PrP deposition was detected in brain. Infectious prion titres were estimated using the method developed by Arnold *et al.* (17). The model uses both the probability of survival (attack rate at each dilution) and the individual mouse incubation periods at each dilution to estimate the infectious load. Infectious titres are given as the most likely value and, in parentheses, the lower and upper limits of the 95% confidence intervals.

Figures:

Figure 1: PMCA amplification of vCJD agent

PMCA reactions were seeded with 1/10 dilution series of a reference vCJD brain homogenate (10% weight / volume -10^{-2} to 10^{-10} dilution). This homogenate has been endpoint titrated by bioassay in bovine PrP expressing mice (tgBov, intracerebral route – $10^{7.7}$ DL_{50}/g).

PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Unseeded reactions were included as specificity control.

PMCA reactions were then submitted to 6 amplification rounds each constituted with 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700.

After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE).

On each gel a scrapie in sheep isolate was used as control (WB control).

Figure 2: vCJD Protein Misfolding Cyclic Amplification in peripheral tissues

PMCA reactions were seeded with ten-fold dilution series of vCJD tissues homogenates (10^{-2} to 10^{-9}) that had been collected post mortem in vCJD-infected patient at clinical stage or preclinical stage of the disease. At least four replicates of each sample dilution were tested in two independent PMCA runs.

Patients (a) vCJD-1, (b) vCJD-2, (c) vCJD-3 and (d) vCJD-4 were at clinical stage of the disease when they deceased. These four patients were homozygotes Methionine at codon 129 of the *PRNP* gene. Patient (e) vCJD-A died while at the preclinical stage of the disease. This patient was heterozygous Methionine/Valine at codon 129 of the *PRNP* gene.

PMCA substrate was prepared using brains from transgenic mice over-expressing the ARQ variant of the sheep prion protein. Unseeded reactions and reaction seeded with tissues from two non-vCJD infected control patients (NC-1 and NC-2 see table 3) were included as specificity control. PMCA reactions were then subjected to 4 amplification rounds each constituted with 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700. After each round, (i) reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round while (ii) a part of the same product was analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} - antibody Sha31 epitope YEDRYYRE).

For each round, the last dilution displaying a positive WB signal in at least half of the tested replicates is indicated on the graph. (○): round 1 - (▽) : round 2 - (△): round 3 - (■): round 4.

Figure 3: PrP^{res} WB detection in PMCA reactions seeded with peripheral tissues

PMCA reactions were seeded with ten-fold dilution series (10^{-2} to 10^{-9}) of vCJD tissues homogenates that had been collected post mortem in vCJD-infected patient at clinical stage (symptomatic vCJD case 1 to vCJD case 4) or preclinical stage of the disease (vCJD asymp.) (see table 2). Reactions seeded with tissues from two non-vCJD patients (see table 2) and unseeded PMCA reactions were included as specificity controls.

Reactions were then subjected to 4 amplification rounds each constituted with 96 cycles (10s sonication-14 minutes and 50 seconds incubation at 39.5°C) in a Qsonica700.

The PMCA reactions were analysed by Western Blot (WB) for the presence of abnormal PK resistant PrP (PrP^{res} -antibody Sha31 epitope YEDRYYRE). On each gel (i) a scrapie in sheep isolate and a vCJD reference isolate were used as control (WB cont.).

For the seven presented tissues (frontal cortex, appendix, lung, liver, salivary gland, pancreas and bone marrow) the dilution of the tissues homogenates used to seed the PMCA reactions is indicated below the immunoblots.

561 **Figure 4: PrP^{res} detection by Western Blot (WB) and Paraffin Embedded Tissue blot**
562 **(PET blot), in the brain of transgenic mice expressing the bovine-PrP (tgBov).**
563 (**a**) PrP^{res} WB of (i) a vCJD isolate (frontal cortex) and of tgBov mice (brain) inoculated with
564 (ii) the same vCJD reference isolate or (iii) with bone marrow samples from vCJD affected
565 patients(vCJD 1 to 4 see table 2) and non vCJD control (NC-1, see table 2). On each gel a
566 scrapie isolate (WB cont.) and a non-inoculated tgBov brain homogenate were included as
567 controls. PrP^{res} immunodetection was carried out using Sha31 monoclonal antibody (epitope:
568 $_{145}$YEDRYYRE$_{152}$) and 12B2 epitope (epitope $_{89}$WGQGG$_{93}$).
569 **(b)** PET blot PrP^{res} distribution in coronal section (thalamus level) of tgBov mice inoculated
570 with a reference vCJD isolate (10% brain homogenate) or bone marrow (10% tissue
571 homogenate) from two vCJD patients at clinical stage of the disease (vCJD-1 and vCJD-3 see
572 table 2). PrP^{res} immunodetection was carried out using Sha31 monoclonal antibody (epitope:
573 $_{145}$YEDRYYRE$_{152}$). Bar: 120µm.
574
575 **Figure 5: Dose-response relationship for the incubation period and probability of**
576 **infection of bovine PrP expressing mice**.
577 Data derived from an endpoint titration of 10% weight/volume frontal cortex homogenate
578 from a vCJD affected patient in tg Bov mice (20µL intracerebral route, see table 1) were used
579 to establish a model that estimate the infectious titre in an homogenate based on the
580 incubation period and the probability of infection in inoculated mice (model is solid line,
581 observed values given by crosses).
582